Cell Journal (Yakhteh)

Guide for Authors

Aims and Scope: Cell Journal (Yakhteh) is a quarterly English publication of Royan Institute of Iran. The aim of the journal is to disseminate information through publishing the most recent scientific research studies on exclusively Cellular, Molecular and other related topics. Cell J, has been certified by Ministry of Culture and Islamic Guidance since 1999 and also accredited as a scientific and research journal by HBI (Health and Biomedical Information) Journal Accreditation Commission since 2000. This journal holds the membership of the Committee on Publication Ethics (COPE).

1. Types of articles

The articles in the field of Fertility and Sterility can be considered for publications in Cell J. These articles are as below:

A. Original articles are scientific reports of the original research studies. The article consists of English Abstract (structured), Introduction, Materials and Methods, Results, Discussion, Conclusion, Acknowledgements, Author's Contributions, and References (**Up to 40**).

B. Review articles are the articles written by well experienced authors and those who have excellence in the related fields. The corresponding author of the review article must be one of the authors of at least three published articles appearing in the references. The review article consists of English Abstract (unstructured), Introduction, Conclusion, Author's Contributions, and References (**Up to 70**).

C. Systematic Reviews

Systematic reviews are a type of literature review that collect and critically analyzes multiple research studies or papers. The Systematic reviews consist of English Abstract (unstructured), Introduction, Materials and Methods, Results, Discussion, Conclusion, Acknowledgements, Author's Contributions, and References (**Up to 70**).

D. Short communications are the articles containing new findings. Submissions should be brief reports of ongoing researches. The short communication consists of English Abstract (unstructured), the body of the manuscript (should not hold heading or subheading), Acknowl-edgements, Author's Contributions, and References (**Up to 30**).

E. Case reports are short discussions of a case or case series with unique features not previously described which make an important teaching point or scientific observation. They may describe novel techniques or use equipment, or new information on diseases of importance. It consists of English Abstracts (Unstructured), Introduction, Case Report, Discussion, Acknowledgements, Author's Contributions, and References (Up to 30).

F. Editorial should be written by either the editor in chief or the editorial board.

G. Imaging in biology should focus on a single case with an interesting illustration such as a photograph, histological specimen or investigation. Color images are welcomed. The text should be brief and informative.

H. Letter to the editors are welcome in response to previously published Cell J articles, and may also include interesting cases that do not meet the requirement of being truly exceptional, as well as other brief technical or clinical notes of general interest.

I. Debate.

2. Submission Process

It is recommended to see the guidelines for reporting different kinds of manuscripts here. This guide explains how to prepare the manuscript for submission. Before submitting, we suggest authors familiarize themselves with **Cell J** format and content by reading the journal via website (www.celljournal.org). The corresponding author ensures that all authors are included in the author list and agree with its order, and they must be aware of the manuscript submission.

A. Author contributions statements

It is essential for authors to include a statement of responsibility in the manuscript that specifies the contribution of every one of them. This participation must include conception and design of the manuscript, data acquisition or data analysis and interpretation, drafting of the manuscript and/or revising it for critically important intellectual content, revision and final approval of the manuscript and statistical analysis, obtaining funding, administrative, technical, or material support, or supervision. Authors who do not meet the above criteria should be acknowledged in the **Acknowledgments Section**.

B. Cover letter

Each article should be accompanied by a cover letter, signed by all authors specifying the following statement: "The manuscript has been seen and approved by all authors and is not under active consideration for publication. It has neither been accepted for publication nor published in another journal fully or partially (except in abstract form). I hereby assign the copyright of the enclosed manuscript to **Cell J.** Corresponding author must confirm the proof of the manuscript before online publishing. Also, is it needed to suggest three peer reviewers in the field of their manuscript.

C. Manuscript preparation

Authors whose first language is not English encouraged to consult a native English speaker in order to confirm his manuscripts to US or British (not a mixture) English usage and grammar. The manuscript should be prepared in accordance with the "International Committee of Medical Journal Editors (ICMJE)". Please send your manuscript in two formats (word and Pdf). The abstract and text pages should have consecutive line numbers in the left margin beginning with title page and continuing through the last page of the written text. Each abbreviation must be defined in the abstract and text when they are mentioned for the first time. Avoid using abbreviation in title. Please use the international and standard abbreviations and symbols.

It should be added that an essential step toward the integration and linking of scientific information reported in published literature is using standardized nomenclature in all fields of science and medicine. Species names must be italicized (e.g., *Homo sapiens*) and also the full genus and species written out in full, both in the title of the manuscript and at the first mention of an organism in a paper.

It is necessary to mention that genes, mutations, genotypes, and alleles must be indicated in italics. Please use the recommended name by consulting the appropriate genetic nomenclature database, e.g., HUGO for human genes. In another words; if it is a human gene, you must write all the letters in capital and italic (e.g., *OCT4, c-MYC*). If not, only write the first letter in capital and italic (e.g., *Oct4, c-Myc*). In addition, protein designations are the same as the gene symbol but are not italicized.

Of note, Cell J will only consider publishing genetic association study papers that are novel and statistically robust. Authors are advised to adhere to the recommendations outlined in the STREGA statement (http://www.strega-statement.org). The following criteria must be met for all submissions:

1. Hardy-Weinberg Equilibrium (HWE) calculations must be carried out and reported along with the P-values if applicable [see Namipashaki et al. 2015 (Cell J, Vol 17, N 2, Pages: 187-192) for a discussion].

2. Linkage disequilibrium (LD) structure between SNPs (if multiple SNPs are reported) must be presented.

3. Appropriate multiple testing correction (if multiple independent SNPs are reported) must be included.

Submissions that fail to meet the above criteria will be rejected before being sent out for review.

Each of the following manuscript components should begin in the following sequence:

Authors' names and order of them must be carefully considered (full name(s), highest awarded academic degree(s), email(s), and institutional affiliation(s) of all the authors in English. Also, you must send mobile number and full postal address of the corresponding author).

Changes to Authorship such as addition, deletion or rearrangement of author names must be made only before the manuscript has been accepted in the case of approving by the journal editor. In this case, the corresponding author must explain the reason of changing and confirm them (which has been signed by all authors of the manuscript). If the manuscript has already been published in an online issue, an erratum is needed.

Title is providing the full title of the research (do not use abbreviations in title).

Running title is providing a maximum of 7 words (no more than 50 characters).

Abstract must include Background, Materials and Methods, Results, and Conclusion (no more than 300 words).

Keywords, three to five, must be supplied by the authors at the foot of the abstract chosen from the Medical Subject Heading (MeSH). Therefore; they must be specific and relevant to the paper.

The following components should be identified after the abstract:

Introduction: The Introduction should provide a brief background to the subject of the paper, explain the importance of the study, and state a precise study question or purpose.

Materials and Methods: It includes the exact methods or observations of experiments. If an apparatus is used, its manufacturer's name and address should be stipulated in parenthesis. If the method is established, give reference but if the method is new, give enough information so that another author can perform it. If a drug is used, its generic name, dose, and route of administration must be given. Standard units of measurements and chemical symbols of elements do not need to be defined.

Statistical analysis: Type of study and statistical methods should be mentioned and specified by any general computer program used.

Ethical considerations: Please state that informed consent was obtained from all human adult participants and from the parents or legal guardians of minors and include the name of the appropriate institutional review board that approved the project. It is necessary to indicate in the text that the maintenance and care of experimental animals complies with National Institutes of Health guidelines for the humane use of laboratory animals, or those of your Institute or agency.

Clinical trial registration: All of the Clinical Trials performing in Iran must be registered in Iranian Registry of Clinical Trials (www. irct.ir). The clinical trials performed abroad, could be considered for publication if they register in a registration site approved by WHO or www.clinicaltrials.gov. If you are reporting phase II or phase III randomized controlled trials, you must refer to the CONSORT Statement for recommendations to facilitate the complete and transparent reporting of trial findings. Reports that do not conform to the CONSORT guidelines may need to be revised before peer reviewing.

Results: They must be presented in the form of text, tables, and figures. Take care that the text does not repeat data that are presented in tables and/or figures. Only emphasize and summarize the essential features of the main results. Tables and figures must be numbered consecutively as appeared in the text and should be organized in separate pages at the end of article while their location should be mentioned in the main text.

Tables and figures: Tables should have a short descriptive heading above them and also any footnotes. Figure's legend should contain a brief title for the whole figure and continue with a short explanation of each part and also the symbols used (no more than 100 words). All figures must be prepared based on cell journal's guideline in color (no more than **6** Figures and Tables) and also in GIF or JPEG format with 300 dpi resolutions.

Supplementary materials would be published on the online version of the journal. This material is important to the understanding

and interpretation of the report and should not repeat material within the print article. The amount of supplementary material should be limited. Supplementary material should be original and not previously published and will undergo editorial and peer review with the main manuscript. Also, they must be cited in the manuscript text in parentheses, in a similar way as when citing a figure or a table. Provide a legend for each supplementary material submitted.

Discussion: It should emphasize the present findings and the variations or similarities with other researches done by other researchers. The detailed results should not be repeated in the discussion again. It must emphasize the new and important aspects of the study.

Conclusion: It emphasizes the new and important aspects of the study. All conclusions are justified by the results of the study.

Acknowledgements: This part includes a statement thanking those who contributed substantially with work relevant to the study but does not have authorship criteria. It includes those who provided technical help, writing assistance and name of departments that provided only general support. You must mention financial support in the study. Otherwise; write this sentence "There is no financial support in this study".

Conflict of Interest: Any conflict of interest (financial or otherwise) and sources of financial support must be listed in the Acknowledgements. It includes providers of supplies and services from a commercial organization. Any commercial affiliation must be disclosed, regardless of providing the funding or not.

References: The references must be written based on the Vancouver style. Thus the references are cited numerically in the text and listed in the bibliography by the order of their appearance. The titles of journals must be abbreviated according to the style used in the list of Journals Indexed in PubMed. Write surname and initials of all authors when there are six or less. In the case of seven or more authors, the names of first six authors followed by "et al." must be listed. The reference of information must be based on the following order:

Article:

Surname(s) and first letter of name & middle name(s) of author(s). Manuscript title. Journal title (abbr).publication date (year); Volume

(Issue): Page number.

Example: Manicardi GC, Bianchi PG, Pantano S, Azzoni P, Bizzaro D, Bianchi U, et al. Presence of endogenous nicks in DNA of ejaculated human spermatozoa and its relationship to chromomycin A3 accessibility. Biol Reprod. 1995; 52(4): 864-867.

Book:

Surname(s) and first letter of name & middle name(s) of author(s). Book title. Edition. Publication place: publisher name; publication date (year); Page number.

Example: Edelman CL, Mandle CL. Health promotion throughout the life span. 2nd ed.ST Louis: Mosby; 1998; 145-163.

Chapter of book:

Surname(s) and first letter of name & middle name(s) of author(s). Chapter title. In: Surname(s) and first letter of name & middle name(s) of editor(s), editors. Book title. Edition. Publication place: publisher name; publication date (year); Page number.

Example: Phillips SJ, Whisnant JP. Hypertension and stroke. In: Laragh JH, Brenner BM, editors. Hypertension: pathophysiology, diagnosis, and management. 2nd ed. New York: Raven Press; 1995; 465-478.

Abstract book:

Example: Nabavi SM. Stem cell therapy for multiple sclerosis. Cell J. 2013; 5 Suppl 1: Os-13.

Thesis:

Name of author. Thesis title. Degree. City name. University. Publication date (year).

Example: Eftekhari Yazdi P. Comparison of fragment removal and co-culture with Vero cell monolayer's on development of human fragmented embryos. Presented for the Ph.D., Tehran. Tarbiyat Modarres University. 2004.

Conferences:

Name(s) of editor(s). Conference title; Holding date; Holding place. Publication place; Publisher name; Publication date (year).

Example: Harnden P, Joffe JK, Jones WG, editors. Germ cell tumors V.Proceedings of the 5th Germ Cell Tumors Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer; 2002.

Internet References

Article:

Surname(s) and first letter of name & middle name(s) of author(s). Manuscript title. Journal title (abbr). Publication date (year); Volume (Issue): Page number. Available from: URL link. (Observation date).

Example: Jahanshahi A, Mirnajafi-Zadeh J, Javan M, Mohammad-Zadeh M, Rohani M. Effect of low-frequency stimulation on adenosine A1 and A2A receptors gene expression in dentate gyrus of perforant path kindled rats. Cell J. 2008; 10 (2): 87-92. Available from: http://www. celljournal.org. (20 Oct 2008).

Book:

Example: Anderson SC, Poulsen KB. Anderson's electronic atlas of hematology.[CD-ROM]. Philadelphia: Lippincott Williams & Wilkins; 2002.

Law:

Example: Embryo donation law. Iran Judicature, Official Gazette of the Islamic Republic of Iran. Available from: http://www.dastour.ir/Brows/?lid=245069.(20 Jul 2013).

D. Proofs are sent by email as PDF files and should be checked and returned within 72 hours of receipt. It is the authors' responsibility to check that all the text and data as contained in the page proofs are correct and suitable for publication. We are requested to pay particular attention to author's names and affiliations as it is essential that these details be accurate when the article is published.

E. Pay for publication: Authors do not have to pay any Manuscript Processing Charge or Open Access Publication Fee. Before publishing author's article, it would be the author's responsibility to pay for the expenses, if the editor feels the level of English used in the manuscript requires editing.

F. Ethics of scientific publication: Manuscripts that have been published elsewhere with the same intellectual material will refer to duplicate publication. If authors have used their own previously published work or work that is currently under review, as the basis for a submitted manuscript, they are required to cite the previous work and indicate how their submitted manuscript offers novel contributions beyond those of the previous work. Research and publication misconduct is considered a serious breach of ethics. The Journal systematically employs iThenticate, a plagiarism detection and prevention software designed to ensure the originality of written work before publication.

Plagiarism of text from a previously published manuscript by the same or another author is a serious publication offence. Some parts of text may be used, only where the source of the quoted material is clearly acknowledged.

3. General information

A. You can send your article via online submission system which is available at our website: **http://www.celljournal.org.** If the article is not prepared according to the format of **Cell J**, it will be returned to authors.

B. The order of article appearance in the Journal is not demonstrating the scientific characters of the authors.

C. Cell J has authority to accept or reject the articles.

D. The received manuscript will be evaluated by associate editor. Cell Journal uses a single-blind peer review system and if the manuscript suits the journal criteria, we select the reviewer. If three reviewers pass their judgments on the manuscript, it will be presented to the editorial board of **Cell J.** If the editorial board has a positive judgment about the manuscript, reviewers' comments will be presented to the corresponding author (the identification of the reviewers will not be revealed). The executive member of journal will contact the corresponding author directly within 3-4 weeks by email. If authors do not receive any reply from journal office after the specified time, they can contact journal office. Finally, executive manager will respond promptly to authors' message.

The Final Checklist

The authors must ensure that before submitting the manuscript for publication, they have to consider the following parts:

1. Title page should contain title, name of the author/coauthors, their academic qualifications, designation & institutions they are affiliated with, mailing address for future correspondence, email address, phone, and fax number.

2. Text of manuscript and References prepared as stated in the "guide for authors" section.

3. Tables should be in a separate page. Figures must be sent in color and also in GIF or JPEG format with 300 dpi resolutions.

4. Covering Letter.

The Editor-in-Chief: Ahmad Hosseini, Ph.D.

Cell Journal (Yakhteh), P.O. Box: 16635-148, Iran Tel/Fax: + 98-21-22510895 Emails: Celljournal@royaninstitute.org

info@celljournal.org

IN THE NAME OF GOD



Gone But not Forgotten

In the memory of the late Director of Royan Institute, Founder of Stem Cells Research in Iran and Chairman of Cell Journal (Yakhteh). May he rest in peace.

Dr. Saeed Kazemi Ashtiani

OWNED: Royan Institute, Iranian Academic Center for Education Culture and Research (ACECR)

CHAIRMAN: Hamid Gourabi, Ph.D., (Professor, Royan Institute, Tehran, Iran)

EDITOR IN CHIEF: Ahmad Hosseini, Ph.D., (Professor, Shahid Beheshti Medical University, Tehran, Iran)

EDITOR ASSOCIATE:

Saeid Abroun, Ph.D., (Associate Professor, Tarbiat Modares University, Tehran, Iran)

EDITORIAL BOARD:

EDITORIAL BOARD: Saeid Abroun, Ph.D. (Associate Professor, Tarbiat Modares University, Tehran, Iran) Karman Alimoghadam, M.D. (Associate Professor, Tehran Medical University, Tehran, Iran) Alireza Asgari, Ph.D., (Professor, Baghyatallah University, Tehran, Iran) Mohammad Kazem Aghaee Mazaheri, D.D.S. (Assistant Professor, ACECR, Tehran, Iran) Gila Behzadi, Ph.D., (Professor, Royan Institute, Tehran, Iran) Mary Familari, Ph.D., (Senior Lecturer, University of Melbourne, Actor, ACECR, Tehran, Iran) Mary Familari, Ph.D., (Professor, Royan Institute, Tehran, Iran) Mary Familari, Ph.D., (Associate Professor, Juniversity of South Carolina, Columbia, USA) Suresh Jesuthasan, Ph.D., (Associate Professor, Juniversity of South Carolina, Columbia, USA) Suresh Jesuthasan, Ph.D., (Professor, Institute of Neurophysiology of University, Tehran, Iran) Saadi Khochbin, Ph.D., (Professor, Institute, Tehresh, Iran) Saadi Khochbin, Ph.D., (Professor, Shahid Beheshti Medical University, Tehran, Iran) Saadi Khochbin, Ph.D., (Professor, Harvard Medical School, USA) Kun Ping Lu, M.D., Ph.D., (Professor, Harvard Medical School, Soston, USA) Navid Marnuchenrabadi, Ph.D., (Ingio Dynamics, Marlborough, USA) Marcos Meseguer, Ph.D., Clinical Embryology Laboratory IVI Valencia, Spain) Seyed Javad Mowia, Ph.D., (Professor, Tarbiat Modares University, Tehran, Iran) Mohammad Hossein Nasi Esfahani, Ph.D., (Professor, Tarbiat Modares University, Tehran, Iran) Mohammad Hossein Sanati, Ph.D., (Professor, Tarbiat Modares University, Tehran, Iran) Mohammad Hossein Sanati, Ph.D., (Professor, Tarbiat Modares University, Tehran, Iran) Mohammad Hossein Sanati, Ph.D., (Professor, Tarbiat Modares University, Tehran, Iran) Mohammad Hossein Sanati, Ph.D., (Institute of Sociate Professor, National Institute, Foran, Iran) Mohammad Hossein Sanati Saeid Abroun, Ph.D., (Associate Professor, Tarbiat Modares University, Tehran, Iran)

EXECUTIVE MANAGER: Farideh Malekzadeh, M.Sc., (Royan Institute, Tehran, Iran)

EXECUTIVE BOARD: Parvaneh Afsharian, Ph.D., (Royan Institute, Tehran, Iran) Reza Azimi, B.Sc., (Royan Institute, Tehran, Iran) Reza Omani-Samani, M.D., (Royan Institute, Tehran, Iran) Elham Amirchaghmaghi, M.D., Ph.D., (Royan Institute, Tehran, Iran) Leila Daliri, M.Sc., (Royan Institute, Tehran, Iran) Mahdi Lotfipanah, M.Sc., (Royan Institute, Tehran, Iran)

ENGLISH EDITOR: Naser Ansari-Pour, Ph.D., (University of Tehran, Tehran, Iran) Saman Eghtesad, Ph.D., (Royan Institute, Tehran, Iran) Vahid Ezzatizadeh, Ph.D., (Royan Institute, Tehran, Iran) Jane Ferrie, Ph.D., (University College of London, London, UK) Mojtaba Nemati, M.Sc., (Royan Institute, Tehran, Iran) Ramin Rezaee, Pharm.D., Ph.D., (Mashhad University of Medical Sciences, Mashhad, Iran) Kim Vagharfard, M.Sc., (Royan Institute, Tehran, Iran) Hamid Zahednasab, M.Sc., (Royan Institute, Tehran, Iran)

GRAPHICS:

Laleh Mirza Ali Shirvani, B.Sc., (Royan Institute, Tehran, Iran)

PUBLISHED & SPONSORED BY: Publication of Royan Institute (ACECR)

Indexed in:

- 1. Thomson Reuters (ISI); Impact Factor: 2.363 2. PubMed
- PubMed Central (PMC)
 National Library Medicine (NLM)
- **Biosis Preview**
- Index Medicus for the Eastern Mediterranean Region (IMEMR) 6.
- Index Copernicus International
 Cambridge Scientific Abstract (CSA)
 EMBASE

- Coopus
 Cinahl Database
 Google Scholar
 Chemical Abstract Service (CAS)

- Chemical Abstract Service (CAS)
 Proquest
 Directory of Open Access Journals (DOAJ)
 Open Academic Journals Index (OAJI)
 Directory of Research Journals Indexing (DRJI)
 Scientific Information Database (SID)
- 19. Iranmedex
- Italimedex
 Regional Information Center for Sciences and Technology (RICeST)
 Islamic World Science Citation Center (ISC)
 Magiran
 Science Library Index

AEEE**R**

Copyright and license information:

The **Cell Journal**_(Yakhteh) is an open access journal which means the articles are freely available online for any individual author to download and use the providing address. The journal is licensed under a Creative Commons Attribution-Non Commercial 3.0 Unported License which allows the author(s) to hold the copyright without restrictions that is permitting unrestricted use, distribution, and reproduction in any medium provided the original work is properly cited.

Editorial Office Address (Dr. Ahmad Hosseini):

Royan Institute, P.O.Box: 16635-148, Tehran, Iran Tel & Fax: (+9821)22510895 Website: www.celljournal.org Emails: info@celljournal.org celljournal@royaninstitute.org

Printing Company:

Jurband Ghaemprint Co. NO. 5, Jalil khoob alley, Niroo Havaei Street, Tehran. Iran



CONTENTS

 Iran's Contribution to Human Proteomic Research 	
Anna Meyfour, Mahya Hosseini, Hamid Sobhanian, Sara Pahlavan	229
Original Articles	
Melatonin Attenuates Upregulation of Duox1 and Duox2 and Protects against Lung Injury following Chest Irrac in Rats	diation
Akbar Aliasgharzadeh, Bagher Farhood, Peyman Amini, Hana Saffar, Elahe Motevaseli, Saeed Rezapoor, Farzad Nouruzi, Dhey Shabeeb, Ahmed Eleojo Musa, Mehran Mohseni, Habiballah Moradi, Masoud Najafi	auldeen 236
• Preliminary Findings of Platelet-Rich Plasma-Induced Ameliorative Effect on Polycystic Ovarian Syndrome Samira Seyyed Anvari, Gholamreza Dehgan, Mazdak Razi	243
• Total Antioxidant Capacity; A Potential Biomarker for Non-Invasive Sex Prediction in Culture Medium of Preimplan Human Embryos	ntation
Nahid Nasiri, Leila Karimian, Fatemeh Hassani, Hamid Gourabi, Hiva Alipour, Zahra Zolfaghari, Poopak Eftekhari-Yazdi	253
Assessment of Short-Term Engraftment Potential of <i>Ex Vivo</i> Expanded Hematopoietic Stem Cells Using Norma Mouse in Utero Transplantation Model Martara Zarrabi Elabah Afral, Mohammad Hassan Aschari, Marziah Ebrahimi	al Fetal
- Autologous Bono Morrow Stom Coll Transplantation in Liver Circhonic offer Correcting Nutritional Anoma	255
• Autologous Bone Marrow Stem Cell Transplantation in Liver Cirriosis after Correcting Nutritional Anoma Controlled Clinical Study Abbas Esmaeilzadeh, Homeira Ommati, Mohammad Mahdi Kooshyar, Lida Jarahi, Kambiz Akhavan Rezayat,Samaneh Saberi, N Vosough Ali Ghassemi	Massoud
Uterine Dendritic Cells Modulation by Mesenchymal Stem Cells Provides A Protective Microenvironment at The Maternal Interface: Improved Pregnancy Outcome in Abortion-Prone Mice Marvam Eskandarian, Seved Mohammad Moazzeni	e Feto- 274
Pluripotency Potential of Embryonic Stem Cell-Like Cells Derived from Mouse Testis Hossein Azizi, Bebruz Asgari, Thomas Skutella	281
a Cons Expression Batterns of Boyan Human Embryonic Stom Colls Correlate with Their Proposity and (201
Serie Expression Patterns of Royan Futhan Embryonic Stein Cens Correlate with Their Propensity and C Systems Hassan Rassouli, Mona Khalaj, Seyedeh-Nafiseh Hassani, Shiva Nemati, Ghasem Hosseini Salekdeh, Hossein Baharvand	290
• Development of An Artificial Male Germ Cell Niche Using Electrospun Poly Vinyl Alcohol/Human Serum All	bumin/
Gelatin Fibers Zahra Borzouie, Majid Naghibzadeh, Ali Reza Talebi, Fatemeh Pourrajab, Ali Jebali, Habib Nikukar, Hosein Molla Hoseini, Khoradmehr, Fatemeh Sadeghian-Nodoushan, Behrouz Aflatoonian, Seyedhossein Hekmatimoghaddam	Arezoo 300
• Stress Hormone and Oxidative Stress Biomarkers Link Obesity and Diabetes with Reduced Fertility Potential Shima Abbasihormozi, Vahab Babapour, Azam kouhkan, Amir Niasari Naslji, Kaveh Afraz, Zahra Zolfaghary, Abdolhossein Shahverdi	307
• Reduction of truncated Kit Expression in Men with Abnormal Semen Parameters, Globozoospermia and His	tory of
Low or Fertilization Failure Somayeh Haghighat, Marziyeh Tavalaee, Zahra Zakeri, Mahdi Noureddini, Abdol-Hossein Shahverdi, Mohammad Hossein Nasr Esfahani	314
 Relationship Study of The Verified Human Epidermal Growth Factor Receptor 2 Amplification with Other Markers and Clinicohistopathological Characteristics in Patients with Invasive Breast Cancer, Using Chromog 	Tumor enic In
Situ Hybridization Abdolazim Sarli, Hossein Mozdarani, Nasser Rakhshani, Sohail Mozdarani	322
• Transcription Levels of nicotinamide nucleotide transhydrogenase and Its Antisense in Breast Cancer Sample Soraya Saleh Gargari, Mohammad Taheri, Vahid Kholghi Oskooei, Mir Davood Omrani, Soudeh Ghafouri-Fard	es 331
 Investigation of Chromosomal Abnormalities and Microdeletion/Microduplication(s) in Fifty Iranian Patient Multiple Congenital Anomalies 	ts with
Akbar Mohammadzadeh, Susan Akbaroghli, Ehsan Aghaei-Moghadam, Nejat Mahdieh, Reza Shervin Badv, Payman Jamali, Kariminejad, Zahra Chavoshzadeh, Saghar Ghasemi Firouzabadi, Roxana Mansour Ghanaie, Ahoura Nozari, Sussan Banihashemi, H Hadipour, Zahra Hadipour, Ariana Kariminejad, Hossein Najmabadi, Yousef Shafeghati, Farkhondeh Behjati	Roxana Fatemeh
• Mitochondrial Polymorphisms, in The D-Loop Area, Are Associated with Brain Tumors Donya Altafi, Soha Sadeghi, Hamed Hojatian, Maryam Torabi Afra, Safoura Pakizeh Kar, Mojtaba Gorji, Massoud Houshmand	350
Short Communication	
• Fermented Garlic Extract Increases Oxygen Consumption and UCP-1 mRNA Expression in Human Adipose-F	Derived

• Front page of Cell Journal_(Yakhteh): Figure 2A-B, Page: 247

Iran's Contribution to Human Proteomic Research

Anna Meyfour, Ph.D.^{1, 2}, Mahya Hosseini, M.Sc.³, Hamid Sobhanian, Ph.D.⁴, Sara Pahlavan, Ph.D.^{3*}

1. Basic and Molecular Epidemiology of Gastrointestinal Disorders Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran 2. Department of Molecular Systems Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology,

ACECR, Tehran, Iran 3. Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and

Technology, ACECR, Tehran, Iran

4. Department of Biology, Payame Noor University, Tehran, Iran

*Corresponding Address: P.O.Box: 16635-148, Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran Email: sarapahlavan@royaninstitute.org

Received: 17/August/2018, Accepted: 17/November/2018

Abstract

Proteomics is a powerful approach to study the whole set of proteins expressed in an organism, organ, tissue or cell resulting in valuable information on physiological or pathological state of a biological system. High throughput proteomic data facilitated the understanding of various biological systems with respect to normal and pathological conditions particularly in the instances of human clinical manifestations. The important role of proteins as the functional gene products encouraged scientists to apply this technology to gain a better understanding of extremely complex biological systems. In last two decades, several proteomics teams have been gradually formed in Iran. In this review, we highlight the most important findings of proteomic research groups in Iran at various areas of stem cells, Y chromosome, infertility, infectious disease and biomarker discovery.

Keywords: Infertility, Iran, Proteomics, Stem Cell

Cell Journal(Yakhteh), Vol 21, No 3, October-December (Autumn) 2019, Pages: 229-235 _

Citation: Meyfour A, Hosseini M, Sobhanian H, Pahlavan S. Iran's contribution to human proteomic research. Cell J. 2019; 21(3): 229-235. doi: 10.22074/ celli.2019.6303.

Introduction

Proteomics is an important field of biological research that studies the whole set of proteins expressed in an organism, organ, tissue or cell resulting in valuable information on physiological or pathological state of a biological system. Thus, it can help the health system via understanding the pathogenesis of diseases (1), discovery of novel biomarkers (2), identification of therapeutic target candidates (3, 4) and drug discovery (5, 6). The application of proteomics in various field of biological sciences, influenced our understanding of molecular mechanisms governing living systems. Thus, many research groups applied this approach to answer multiple unsolved biological/clinical questions.

During last two decades several proteomics teams have been gradually formed in Iran and, exploited proteomics in various human research fields including stem cell science, infertility, biomarker discovery and infectious disease. Owing to significant progress in stem cell proteomics, it has been one of the most active filed of proteomics studies in Iran (7). In 2005, Iranian scientist generated the first proteome map of human embryonic stem cell membrane (hESC) (8). Since then, many studies have been performed to identify proteins involved in stem cell differentiation as well as lineage specific cell surface markers (9).

Iranian Scientists have also contributed in several international projects. These include Human Y Chromosome Proteome Project and Asia Oceania Human Proteome Organization Initiative on hESC membrane proteome. This review highlights the most important

findings of proteomic research groups in Iran at various areas of stem cells, infertility, infectious disease and biomarker discovery (Table 1).

Proteomic research in Iran

Stem cell proteomics

Over last two decades, stem cell institutes of Iran have attracted major funding and recruited scientists to accelerate the development of this field, in order to improve both developmental knowledge and clinical translation for the purpose of regenerative medicine (7, 10).

hESCs are pluripotent cells that have the ability for selfrenewal and differentiation to all three embryonic germ layers. They provide an unprecedented source of cells for developmental study, disease modeling, and drug screening as well as cell-based therapies. Scientists have expressed tremendous interest in the molecular mechanisms that govern pluripotency, proliferation, and differentiation of ESCs, and employed various approaches to unravel these regulatory mechanisms (11). In Iran, proteomics has been used to study molecular mechanisms which play a role in maintaining the undifferentiated state of hESCs and induced pluripotent stem cells (iPSCs) (8, 12) as well as differentiation of monkey, mouse, and human ESC lines into embryoid bodies (13-16) and hESC neural differentiation (17, 18). The proteome of spinal cords in healthy and experimental autoimmune encephalomyelitis (EAE) samples have been analyzed before and after transplantation of ESC-derived neural precursors (19).

	Table 1: An overview to human proteomic research in Iran					
	Field	Finding	Proteomic Tool	Reference		
1	Stem cell	Molecular mechanisms which play a role in maintaining the undifferentiated state of hESCs and iPSCs	2-DE-MS/MS 2-DE-MS/MS	(8) (12)		
2		Molecular mechanisms which play a role in differentiation of mouse, monkey, and human ESC lines into embryoid bodies	2-DE-MS/MS 2-DE-MS/MS iTRAQ-MS/MS 2-DE-MS/MS	(13) (14) (15) (16)		
3		Molecular mechanisms which play role in neural differentiation of ESC	2D-DIGE-MS/MS	(17)		
4		Molecular mechanisms of EAE recovery after transplantation of ESC-derived neural precursors	2-DE-MS/MS	(19)		
5		Identification of cytosolic and membrane associated protein complexes of hESC	Blue native PAGE-MS/MS	(20)		
6		Reduction of focal adhesion enables the maintenance of undifferentiated and pluripotent states of mESCs	Label-free mass spectrometry	(21) (22)		
7		Identification of hundreds of organelle specific proteins and functional assignment of three novel membrane proteins to pluripotency	Label-free mass spectrometry	(23)		
8		Deep subcellular proteomics resulted in identification of 15, 3, 13 gold missing proteins in membrane, cytoplasm, and nucleus of hESCs	Label-free mass spectrometry	(24)		
9		Identification of ALCAM as a specific surface marker to enrich ISL1 ⁺ cardiac progenitor cells	Label-free mass spectrometry	(25)		
10		Identification of two novel cell surface markers in order to purify Embryonic Dopamine progenitor cells	Label-free mass spectrometry	(26)		
11	The Y Chromosome Human Proteome Project (Y-HPP)	Identification of two MPs (XKRY and CYORF15A) and introduction of promising molecular markers to predict retrievable sperm presence in MA patients	Western blot/Immmunohistochemistry	(27)		
12		Identification of two novel splice variants of KDM5D and their possible role in prostate cancer cells' growth	Label-free mass spectrometry	(28)		
13		Identification of DDX3Y role in neural differentiation of NTERA-2	Label-free mass spectrometry	(29)		
14		Identification of a missing protein, TBL1Y, and its role in cardiac differentiation of hESCs	Western blot/Immmunofluorescent	(30)		
15	Infertility	Identification of proteins which are differentially expressed in spermatozoa of men with or without varicocele	2-DE-MS/MS	(31)		
16		Identification of proteins which are differentially expressed in spermatozoa of patients with grade 3 varicocele before and after varicocelectomy	2-DE-MS/MS	(32)		
17		Identification of 14 differentially expressed proteins in sperm tails of asthenozoospermia patients compared to normozoospermia men	2-DE-MS/MS	(33)		
18		Identification of anti-sperm antibody targets in azoosprmic men	2-DE-MS/MS	(34)		
19		Identification of novel candidate proteins which play role in regulation of spermatogenesis	Label-free mass spectrometry	(35)		

Table 1: Continued					
	Field	Finding	Proteomic Tool	Reference	
20		Reconstruction of SpermNet	Whole-proteome data and the mCADRE algorithm	(36)	
21		Identification of some proteins in endometrial tissue of PCOS patients which play important roles in fecundity and fecundability	2-DE-MS/MS	(37)	
22	Infectious disease	Identification of proteins which play important roles in rabies virus pathogenesis	2-DE-MS/MS	(1, 38-40)	
23		Proteomics of Leishmaniasis	2-DE-MS/MS	(41, 42)	
24	Biomarker discovery	Introduction of a panel of urinary prognostic biomarkers for classification of IgA nephropathy	Label-free mass spectrometry	(43)	
25		Identification of a panel of biomarkers to predict the responsiveness to steroid therapy in focal segmental glomerulosclerosis	Label-free mass spectrometry	(44)	
26		Identification of a panel of biomarkers for early diagnosis as well as sensitivity to dexamethasone therapy in B cell acute lymphoblastic leukemia	2-DE-MS/MS	(45)	
27		Introduction of Ig Kappa Chain C region (IGKC) as a potential biomarker for fluoxetine responsiveness and patient follow up	2-DE-MS/MS	(46)	

Subcellular localization of a protein to different organelles can greatly determine the decisive protein function (47). Blue native polyacrylamide gel electrophoresis (PAGE) was used to identify cytoplasmic and membrane-associated complexes in hESCs (20).

The shotgun proteome approach was used to study ground state pluripotency. The results indicated that reduced focal adhesion enabled mouse embryonic stem cells (mESCs) to be maintained in undifferentiated and pluripotent states (21, 22).

Asia Oceania Human Proteome Organization Initiative on hESC membrane proteome

Iranian scientists are actively involved in hESC Membrane Proteome Initiative, an Asia Oceania Human Proteome Organization (AOHUPO) initiative Chaired by Prof. Ghasem Hosseini Salekdeh from Iran and Prof. Yu Ju Chen from Taiwan.

Although embryonic stem cells have become a cell of choice for multiple purposes from developmental studies to cell-based therapeutics, there are still unknown remaining aspects of these cells, such as a complete protein map of their membrane proteins. Researchers undertook a study on subcellular proteomics of hESC fractions using a TripleTOF mass spectrometer as part of the AOHUPO and projects related to the hESC Membrane Proteome Initiative. This study resulted in the identification of hundreds of organelle specific proteins and was followed by functional assignment of three novel membrane proteins to pluripotency (23).

In 2018, the most comprehensive proteome map of hESC has been published as a result of this initiative. A deep subcellular proteomics approach was used to identify the membrane, cytoplasmic and nuclear proteins of hESCs in survey for identification of missing proteins (MPs). This great study revealed 15, 3, 13 gold missing MPs in membrane, cytoplasm, and nucleus fractions which determined to play role in self-renewal, regulation of differentiation, epigenetic regulations, and cellular layers development in hESCs (24).

This initiative also tuned the focus of proteomic studies toward discovering cell surface markers for the purpose of lineage-specific cell sorting. LIM-homeodomain transcription factor ISL1 is one of the main markers of cardiac progenitor cells that is believed to be the master regulator of fate determination for the secondary heart field-derived cardiac cells (48-52). To purify a population of cardiac progenitor cells, ISL1 can not be directly used due to its nuclear localization. Thus, a genetic selection strategy was used to mark ISL1⁺cells in order to identify a cell surface marker by label-free shotgun proteomics approach for future applications in safe clinical sorting of cardiac lineage-specific cells. ALCAM (CD166) was introduced as a cell surface marker which could successfully purify the population of ISL1+ cardiac progenitor cells with the ability to recover cardiac function

and improve angiogenesis in a rat model of myocardial infarction (25).

In a similar approach, a transcription factor that marks early dopaminergic neurons, LIM homeobox transcription factor 1 alpha (LMX1A), was used to generate a knockin GFP reporter human embryonic stem cell (hESC) line in order to purify this particular neuronal population for further cell surface marker identification. Using shotgun proteomics, Fathi et. al. introduced two cell surface markers, polysialylated embryonic form of neural cell adhesion molecule (PSA-NCAM) and contactin 2 (CNTN2), which could successfully enrich LMX1A⁺ progenitor cells. Further transplantation of CNTN2⁺ cells improved Parkinson's disease-related phenotypes in rat models (26).

Iran's contribution to human proteome project: the Y chromosome human proteome project

The Y chromosome human proteome project (Y-HPP) is being conducted in Iran (53). The project focuses on identification of MPs and study of the function of proteins and their association with diseases (54, 55). Rastegar and colleagues performed an isoform-level gene expression profiling of Y chromosome genes within the azoospermia factor (AZF) regions, their X counterparts, and a few autosomal paralogues in four different groups: healthy individuals with preserved spermatogenesis, patients with non-obstructive azoospermia (NOA), Sertoli-cellonly syndrome (SCOS), and premeiotic maturation arrest (MA). They identified, for the first time, two MPs (XKRY and CYORF15A). Rastegar introduced HSFY1-3, RBMX2, BPY2-1, DAZL-1, and KDM5C2 as promising molecular markers to predict retrievable sperm presence in MA patients (27). Jangravi et al. (28) studied KDM5D expression, an MSY gene, in a prostate cancer cell line (DU-145). They found two novel splice variants with lengths of 2650 bp and 2400 bp. Knockdown of these two variants resulted in higher growth and lower apoptosis rate of prostate cancer cells.

Shotgun label-free quantitative proteomics revealed alterations in abundance of proteins involved in RNA processing, protein synthesis, apoptosis, the cell cycle, growth, and proliferation in KDM5D knockdown cells. Vakilian et al. (29) investigated the expression of 23 MSY genes and 15 of their X homologues during neural cell differentiation of NTERA-2 human embryonal carcinoma cell line (NT2) cells. They observed alterations in expression of several MSY genes, from which DDX3Y was knocked down to further investigate its function during neurogenesis. Label-free quantitative shotgun proteomics showed that DDX3Y knockdown resulted in expression alterations of proteins involved in the cell cycle, RNA splicing, and apoptosis. They suggested that DDX3Y might play a multifunctional role in neural cell development. Meyfour and co-workers studied the proteome of Y chromosome during cardiogenesis of hESCs. They observed alterations in the expression and protein localization of some of the MSY genes

during cardiomyocyte differentiation of hESCs from which TBL1Y, a MP, was knocked down for further functional analysis. TBL1Y knockdown resulted in inefficient cardiac differentiation of hESCs along with generation of cardiomyocytes with impaired contraction (30).

Proteomics in infertility research

Human male infertility accounts for approximately 7-10% of infertilities (56) and is an important medical issue with an unknown etiology in most cases. Hosseinifar et al. have compared the sperm protein profiles of men with and without varicocele by Two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS). Their results indicated that heat shock, mitochondrial, and cytoskeletal proteins were mainly affected by varicocele (31). In another study, they analyzed the proteome profile from patients with varicocele and poor sperm quality before and after varicocelectomy. Their findings showed that the altered proteins played a key role in sperm production, protection of DNA integrity, and sperm motility (32). In a study by Hashemitabar et al. (33), the proteome profile of the sperm tail was analyzed in patients with normozoospermia and asthenozoospermia. For the first time, they identified four differentially expressed proteins which could be potential markers to better diagnose sperm dysfunction, new contraceptive targets, and prediction of embryo quality.

Zangbar et al. (34) profiled the sperm proteins and blotted them with sera of healthy fertile or obstructive azoospermia (OA) in order to explore the anti-sperm protein targets in azoospermic men. They observed that sera from OA patients might contain antibodies against two sperm proteins, Tektin-2 and triose phosphate isomerase. Alikhani et al. (35) performed shotgun proteomics in order to unravel the molecular mechanisms involved in male azoospermia. They compared the protein profile of testicular tissues from OA patients and NOA that included MA and SCOS. Their findings introduced novel candidate proteins, which included key transcription factors associated with azoospermia. Recently, Asghari et al. (36) reconstructed the first proteome-scale model of the sperm cell by using whole-proteome data and the Metabolic Context-specificity Assessed by Deterministic Reaction Evaluation (mCADRE) algorithm. Their model could predict the novel non-glycolytic genes for deficient energy metabolism in addition to known pathways in asthenozoospermia. Proteomics was employed to address infertility complications in female reproductive system, too. Proteome profile of endometrial tissue in polycystic ovary syndrome (PCOS) was compared to healthy fertile women and resulted in identification of 70 proteins which assigned a role in oxidative stress, inflammation, apoptosis and the cytoskeletal rearrangement that may underlie impaired fecundability and low pregnancy rate in PCOS women (37).

Proteomics in infectious disease

Proteomics, as a novel approach, has helped the

scientific community to develop a molecular picture of infectious diseases and their spread, which will definitely result in better control of disease prevalence and the development of more efficient therapies (57). Rabies is a neurodegenerative disease caused by a life threatening rabies virus. Proteomics has been applied to study the effect of the virus on baby hamster kidney cells (BHK-21) (1), a neuroblastoma cell line (38), lymphocytes of infected mice (39), and human brain infected by the virus (40). Leishmaniasis is another infectious disease studied by proteomics (41, 42). More than 20 species of intracellular parasites that belong to the Leishmania genus cause this infectious disease. The symptoms range from simple self-limiting cutaneous ulcers to sever disfiguring mucocutaneous lesions, and even fatal visceral disease. Among these infectious diseases, the diagnosis and treatment of viscerotropic leishmaniasis appears challenging. It is anticipated that technologies developed in the course of C-HPP can be applied for research of infectious diseases in the future.

Proteomics in biomarker discovery

Despite great advances in our understanding of epidemiology and pathophysiology of diseases, but diagnosis and therapeutic decisions for many pathological conditions rely on invasive tools. Moreover, prognosis or early detection of few disease conditions are possible by biomarker discovery. Therefore, great efforts are made to introduce ideal biomarkers to improve prognosis, diagnosis and predictive response to treatment. Advances in proteomic technologies can greatly influence the field of biomarker discovery (58). This concept has been the objective of some research projects in Iran.

The classification of immunoglobulin A (IgA) nephropathy using scoring systems showed inconsistencies among nephrologists. Kalantari et al introduced a panel of prognostic biomarkers using liquid chromatography tandem-mass spectrometry (LC-MS/ MS) which can be applied for prognosis and classification of IgA nephropathy (43). Furthermore, they found a panel of biomarkers using nano-LC-MS/MS which could help to predict the responsiveness to steroid therapy in focal segmental glomerulosclerosis (44). Dehghan-Nayeri et al. (45) identified CAPZA1, CAPZB, CLIC1, PNP and PSME1 as a panel of biomarkers for early diagnosis as well as sensitivity to dexame has one therapy in B cell acute lymphoblastic leukemia. Zamanian Azodi et al. (46) studied proteome profile of obsessive-compulsive disorder (OCD) patients before and after treatment with fluoxetine and introduced Ig Kappa Chain C region (IGKC) as a potential biomarker for fluoxetine responsiveness and patient follow up.

Proteomics in the mirror of global endeavor

Iranian researchers have significantly contributed to proteomic science by establishing international collaborations. They have also pioneered some research areas such as the proteome map of human embryonic stem cells. Furthermore, Iran has contributed in C-HPP as the country responsible for the proteome profile of Y chromosome and has successfully fulfilled this goal by identification of multiple Y chromosome MPs. Iranian proteomic researchers developed a new strategy for identification of MPs with highly similar homologous proteins where MS cannot provide sufficient data. Furthermore, introduction of novel surface markers for pluripotent stem cells and lineage committed cells was another important achievement of proteomic research in Iran which may facilitate the clinical translation of these cells. Moreover, recently neuroproteomics (59, 60), post translational modification (PTM) analysis (61) as well as functional characterization of identified Y chromosome proteins with no known function as 'dark proteins' (62) gained more attention in Iran. This influential role of Iran will be continued to the future of proteomics as more international collaborations are established and higher number of motivated researchers get involved.

Conclusion

In line with global advancement of proteomic research, Iranian scientists have been contributing to this research area by using various techniques of protein analysis. Despite limitations in high technology equipment required for proteomic studies, such as MS, this contribution continued to be in effect by establishing alternative experimental tools as well as forming international collaborations. These universal efforts have resulted in and will continue to provide valuable information directly or indirectly empowering health system by understanding the pathogenesis of diseases, discovery of novel biomarkers, identification of therapeutic target candidates and drug discovery.

Acknowledgements

We are grateful to Dr. Ghasem Hosseini Salekdeh for all his help and support. This study was supported by a grant from Royan Institute. The authors declare no conflict of interest.

Authors' Contributions

A.M.; Contributed to explicit literature search and classification as well as summarizing papers and writing manuscript. M.H.; Contributed to classification of papers and table preparation. H.S., S.P.; Contributed to manuscript writing and revision. S.P.; Supervised the study. All authors read and approved the final manuscript.

References

- Zandi F, Eslami N, Soheili M, Fayaz A, Gholami A, Vaziri B. Proteomics analysis of BHK-21 cells infected with a fixed strain of rabies virus. Proteomics. 2009; 9(9): 2399-2407.
- Ray S, Reddy PJ, Jain R, Gollapalli K, Moiyadi A, Srivastava S. Proteomic technologies for the identification of disease biomarkers in serum: advances and challenges ahead. Proteomics. 2011; 11(11): 2139-2161.
- 3. Moseley FL, Bicknell KA, Marber MS, Brooks G. The use of proteomics to identify novel therapeutic targets for the treatment of

disease. J Pharm Pharmacol. 2007; 59(5): 609-6028.

- Ardekani AM, Maghsudi N, Meyfour A, Ghasemi R, Lakpour N, Nooshinfar E, et al. Stress-induced proteomic changes in the hippocampus of pregnant wistar rats. Avicenna J Med Biotechnol. 2011; 3(4): 157-166.
- 5. Burbaum J, Tobal GM. Proteomics in drug discovery. Curr Opin Chem Biol. 2002; 6(4): 427-433.
- Meyfour A, Rezaei-Tavirani M. The role of omics in clinical and pharmaceutical researches. J Mazandaran Univ Med Sci. 2018; 28(161): 166-184.
- Miremadi T, Salekdeh GH, Aghdami N, Gharanfoli M, Vasei M, Kouhkan A, et al. Stem cell research and therapy in the Islamic republic of Iran: pioneering in the Islamic world. Stem Cells Dev. 2013; 22(1): 51-57.
- Baharvand H, Hajheidari M, Ashtiani SK, Salekdeh GH. Proteomic signature of human embryonic stem cells. Proteomics. 2006; 6(12): 3544-3549.
- Reiland S, Salekdeh GH, Krijgsveld J. Defining pluripotent stem cells through quantitative proteomic analysis. Expert Rev Proteomics. 2011; 8(1): 29-42.
- Gheisari Y, Baharvand H, Nayernia K, Vasei M. Stem cell and tissue engineering research in the Islamic republic of Iran. Stem Cell Rev. 2012; 8(3): 629-639.
- Baharvand H, Fathi A, van Hoof D, Salekdeh GH. Concise review: trends in stem cell proteomics. Stem Cells. 2007; 25(8): 1888-1903.
- Faradonbeh MZ, Gharechahi J, Mollamohammadi S, Pakzad M, Taei A, Rassouli H, et al. An orthogonal comparison of the proteome of human embryonic stem cells with that of human induced pluripotent stem cells of different genetic background. Mol Biosyst. 2012; 8(6): 1833-1840.
- Baharvand H, Fathi A, Gourabi H, Mollamohammadi S, Salekdeh GH. Identification of mouse embryonic stem cell-associated proteins. J Proteome Res. 2008; 7(1): 412-423.
- Nasrabadi D, Rezaei Larijani M, Pirhaji L, Gourabi H, Shahverdi A, Baharvand H, et al. Proteomic analysis of monkey embryonic stem cell during differentiation. J Proteome Res. 2009; 8(3): 1527-1539.
- Jadaliha M, Lee HJ, Pakzad M, Fathi A, Jeong SK, Cho SY, et al. Quantitative proteomic analysis of human embryonic stem cell differentiation by 8-plex iTRAQ labelling. PLoS One. 2012; 7(6): e38532.
- Fathi A, Pakzad M, Taei A, Brink TC, Pirhaji L, Ruiz G, et al. Comparative proteome and transcriptome analyses of embryonic stem cells during embryoid body-based differentiation. Proteomics. 2009; 9(21): 4859-4870.
- Fathi A, Hatami M, Vakilian H, Han CL, Chen YJ, Baharvand H, et al. Quantitative proteomics analysis highlights the role of redox hemostasis and energy metabolism in human embryonic stem cell differentiation to neural cells. J Proteomics. 2014; 101: 1-16.
- Fathi A, Hatami M, Hajihosseini V, Fattahi F, Kiani S, Baharvand H, et al. Comprehensive gene expression analysis of human embryonic stem cells during differentiation into neural cells. PLoS One. 2011; 6(7): e22856.
- Fazeli AS, Nasrabadi D, Pouya A, Mirshavaladi S, Sanati MH, Baharvand H, et al. Proteome analysis of post-transplantation recovery mechanisms of an EAE model of multiple sclerosis treated with embryonic stem cell-derived neural precursors. J Proteomics. 2013; 94: 437-450.
- Shekari F, Taei A, Pan TL, Wang P-W, Baharvand H, Salekdeh GH. Identification of cytoplasmic and membrane-associated complexes in human embryonic stem cells using blue native PAGE. Mol Biosyst. 2011; 7(9): 2688-2701.
- Taleahmad S, Mirzaei M, Parker LM, Hassani SN, Mollamohammadi S, Sharifi-Zarchi A, et al. Proteome analysis of ground state pluripotency. Sci Rep. 2015; 5: 17985.
- Taleahmad S, Mirzaei M, Samadian A, Hassani SN, Haynes PA, Salekdeh GH, et al. Low focal adhesion signaling promotes ground state pluripotency of mouse embryonic stem cells. J Proteome Res. 2017; 16(10): 3585-3595.
- Shekari F, Nezari H, Larijani MR, Han CL, Baharvand H, Chen YJ, et al. Proteome analysis of human embryonic stem cells organelles. J Proteomics. 2017; 162: 108-118.
- Weldemariam MM, Han CL, Shekari F, Kitata RB, Chuang CY, Hsu WT, et al. Subcellular proteome landscape of human embryonic stem cells revealed missing membrane proteins. J Proteome Res. 2018.
- Ghazizadeh Z, Fattahi F, Mirzaei M, Bayersaikhan D, Lee J, Chae S, et al. Prospective isolation of isl1(+) cardiac progenitors from human escs for myocardial infarction therapy. Stem Cell Reports. 2018; 10(3): 848-859.

- Fathi A, Mirzaei M, Dolatyar B, Sharifitabar M, Bayat M, Shahbazi E, et al. Discovery of Novel Cell Surface Markers for Purification of Embryonic Dopamine Progenitors for Transplantation in Parkinson's Disease Animal Models. Mol Cell Proteomics. 2018; 17(9): 1670-1684.
- Ahmadi Rastegar D, Sharifi Tabar M, Alikhani M, Parsamatin P, Sahraneshin Samani F, Sabbaghian M, et al. Isoform-level gene expression profiles of human Y Chromosome azoospermia factor genes and their X chromosome paralogs in the testicular tissue of non-obstructive azoospermia patients. J Proteome Res. 2015; 14(9): 3595-3605.
- Jangravi Z, Tabar MS, Mirzaei M, Parsamatin P, Vakilian H, Alikhani M, et al. Two splice variants of Y chromosome-located lysine-specific demethylase 5D Have distinct function in prostate cancer cell line (DU-145). J Proteome Res. 2015; 14(9): 3492-3502.
- Vakilian H, Mirzaei M, Sharifi Tabar M, Pooyan P, Habibi Rezaee L, Parker L, et al. DDX3Y, a male-specific region of Y Chromosome gene, may modulate neuronal differentiation. J Proteome Res. 2015; 14(9): 3474-3483.
- Meyfour A, Ansari H, Pahlavan S, Mirshahvaladi S, Rezaei-Tavirani M, Gourabi H, et al. Y chromosome missing protein, TBL1Y, May play an important role in cardiac differentiation. J Proteome Res. 2017; 16(12): 4391-4402.
- Hosseinifar H, Gourabi H, Salekdeh GH, Alikhani M, Mirshahvaladi S, Sabbaghian M, et al. Study of sperm protein profile in men with and without varicocele using two-dimensional gel electrophoresis. Urology. 2013; 81(2): 293-300.
- Hosseinifar H, Sabbaghian M, Nasrabadi D, Modarresi T, Dizaj AV, Gourabi H, et al. Study of the effect of varicocelectomy on sperm proteins expression in patients with varicocele and poor sperm quality by using two-dimensional gel electrophoresis. J Assist Reprod Genet. 2014; 31(6): 725-729.
- Hashemitabar M, Sabbagh S, Orazizadeh M, Ghadiri A, Bahmanzadeh M. A proteomic analysis on human sperm tail: comparison between normozoospermia and asthenozoospermia. J Assist Reprod Genet. 2015; 32(6): 853-863.
- Zangbar MS, Keshtgar S, Zolghadri J, Gharesi-Fard B. Antisperm protein targets in azoospermia men. J Hum Reprod Sci. 2016; 9(1): 47-52.
- Alikhani M, Mirzaei M, Sabbaghian M, Parsamatin P, Karamzadeh R, Adib S, et al. Quantitative proteomic analysis of human testis reveals system-wide molecular and cellular pathways associated with non-obstructive azoospermia. J Proteomics. 2017; 162: 141-154.
- Asghari A, Marashi SA, Ansari-Pour N. A sperm-specific proteomescale metabolic network model identifies non-glycolytic genes for energy deficiency in asthenozoospermia. Syst Biol Reprod Med. 2017; 63(2): 100-112.
- Amjadi F, Mehdizadeh M, Ashrafi M, Nasrabadi D, Taleahmad S, Mirzaei M, et al. Distinct changes in the proteome profile of endometrial tissues in polycystic ovary syndrome compared with healthy fertile women. Reprod Biomed Online. 2018; 37(2): 184-200.
- Zandi F, Eslami N, Torkashvand F, Fayaz A, Khalaj V, Vaziri B. Expression changes of cytoskeletal associated proteins in proteomic profiling of neuroblastoma cells infected with different strains of rabies virus. J Med Virol. 2013; 85(2): 336-347.
- Vaziri B, Torkashvand F, Eslami N, Fayaz A. Comparative proteomics analysis of mice lymphocytes in early stages of infection by different strains of rabies virus. Indian J Virol. 2012; 23(3): 311-316.
- Farahtaj F, Zandi F, Khalaj V, Biglari P, Fayaz A, Vaziri B. Proteomics analysis of human brain tissue infected by street rabies virus. Mol Biol Rep. 2013; 40(11): 6443-6450.
- Hajjaran H, Mousavi P, Burchmore R, Mohebali M, Mohammadi Bazargani M, Salekdeh GH, et al. Comparative Proteomic Profiling of leishmania tropica: investigation of a case Infected with simultaneous cutaneous and viscerotropic leishmaniasis by 2-dimentional electrophoresis and mass spectrometry. Iran J Parasitol. 2015; 10(3): 366-380.
- 42. Hajjaran H, Mohammadi Bazargani M, Mohebali M, Burchmore R, Salekdeh GH, Kazemi-Rad E, et al. Comparison of the Proteome profiling of iranian isolates of leishmania tropica, L. major and L. infantum by two-dimensional electrophoresis (2-DE) and Massspectrometry. Iran J Parasitol. 2015; 10(4): 530-540.
- 43. Kalantari S, Rutishauser D, Samavat S, Nafar M, Mahmudieh L, Rezaei-Tavirani M, et al. Urinary prognostic biomarkers and classification of IgA nephropathy by high resolution mass spectrometry coupled with liquid chromatography. PLoS One. 2013; 8(12): e80830.
- 44. Kalantari S, Nafar M, Rutishauser D, Samavat S, Rezaei-Tavirani

M, Yang H, et al. Predictive urinary biomarkers for steroid-resistant and steroid-sensitive focal segmental glomerulosclerosis using high resolution mass spectrometry and multivariate statistical analysis. BMC Nephrol. 2014; 15: 141.

- Dehghan-Nayeri N, Eshghi P, Pour KG, Rezaei-Tavirani M, Omrani MD, Gharehbaghian A. Differential expression pattern of protein markers for predicting chemosensitivity of dexamethasone-based chemotherapy of B cell acute lymphoblastic leukemia. Cancer Chemother Pharmacol. 2017; 80(1): 177-185.
- Zamanian Azodi M, Rezaei Tavirani M, Arefi Oskouie A, Hamdieh M, Derakhshan MK, Ahmadzadeh A, et al. Fluoxetine regulates Ig kappa chain c region expression levels in the serum of obsessivecompulsive disorder patients: a proteomic approach. Iran J Pharm Res. 2017; 16(3): 1264-1271.
- Shekari F, Baharvand H, Salekdeh GH. Organellar proteomics of embryonic stem cells. Adv Protein Chem Struct Biol. 2014; 95: 215-230.
- Buckingham M, Meilhac S, Zaffran S. Building the mammalian heart from two sources of myocardial cells. Nat Rev Genet. 2005; 6(11): 826-835.
- Cai CL, Liang X, Shi Y, Chu PH, Pfaff SL, Chen J, et al. Isl1 identifies a cardiac progenitor population that proliferates prior to differentiation and contributes a majority of cells to the heart. Dev Cell. 2003; 5(6): 877-889.
- Laugwitz KL, Moretti A, Lam J, Gruber P, Chen Y, Woodard S, et al. Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. Nature. 2005; 433(7026): 647-653.
- Liang X, Zhang Q, Cattaneo P, Zhuang S, Gong X, Spann NJ, et al. Transcription factor ISL1 is essential for pacemaker development and function. J Clin Invest. 2015; 125(8): 3256-3268.
- Zhou B, Ma Q, Rajagopal S, Wu SM, Domian I, Rivera-Feliciano J, et al. Epicardial progenitors contribute to the cardiomyocyte lineage in the developing heart. Nature. 2008; 454(7200): 109-113.
- 53. Meyfour A, Pahlavan S, Sobhanian H, Salekdeh GH. 17th Chro-

mosome-Centric Human Proteome Project Symposium in Tehran. Proteomics. 2018; 18(7): e1800012.

- Jangravi Z, Alikhani M, Arefnezhad B, Sharifi Tabar M, Taleahmad S, Karamzadeh R, et al. A fresh look at the male-specific region of the human Y chromosome. J Proteome Res. 2013; 12(1): 6-22.
- Meyfour A, Pooyan P, Pahlavan S, Rezaei-Tavirani M, Gourabi H, Baharvand H, et al. Chromosome-centric human proteome project allies with developmental biology: a case study of the role of Y chromosome genes in organ development. J Proteome Res. 2017; 16(12): 4259-4272.
- Moore FL, Reijo-Pera RA. Male sperm motility dictated by mother's mtDNA. Am J Hum Genet. 2000; 67(3): 543-548.
- 57. Greco TM, Cristea IM. Proteomics tracing the footsteps of infectious disease. Mol Cell Proteomics. 2017; 16(4 suppl 1): S5-S14.
- Crutchfield CA, Thomas SN, Sokoll LJ, Chan DW. Advances in mass spectrometry-based clinical biomarker discovery. Clin Proteomics. 2016; 13: 1.
- Mirzaei M, Gupta VB, Chick JM, Greco TM, Wu Y, Chitranshi N, et al. Age-related neurodegenerative disease associated pathways identified in retinal and vitreous proteome from human glaucoma eyes. Sci Rep. 2017; 7(1): 12685.
- Sabbagh U, Monavarfeshani A, Su K, Zabet-Moghadam M, Cole J, Carnival E, et al. Distribution and development of molecularly distinct perineuronal nets in visual thalamus. J Neurochem. 2018; 147(5): 626-646.
- Byun K, Yoo Y, Son M, Lee J, Jeong GB, Park YM, et al. Advanced glycation end-products produced systemically and by macrophages: a common contributor to inflammation and degenerative diseases. Pharmacol Ther. 2017; 177: 44-55.
- Paik YK, Lane L, Kawamura T, Chen YJ, Cho JY, LaBaer J, et al. Launching the C-HPP pilot project for functional characterization of identified proteins with no known function. J Proteome Res (ahead of print).

Melatonin Attenuates Upregulation of *Duox1* and *Duox2* and Protects against Lung Injury following Chest Irradiation in Rats

Akbar Aliasgharzadeh, Ph.D.¹, Bagher Farhood, Ph.D.¹, Peyman Amini, M.Sc.², Hana Saffar, Ph.D.³, Elahe

Motevaseli, Ph.D.⁴, Saeed Rezapoor, M.Sc.², Farzad Nouruzi, M.Sc.⁵, Dheyauldeen Shabeeb, Ph.D.^{6, 7}, Ahmed

Eleojo Musa, Ph.D.^{6, 8}, Mehran Mohseni, Ph.D.¹, Habiballah Moradi, Ph.D.¹, Masoud Najafi, Ph.D.^{9*}

Departments of Medical Physics and Radiology, Faculty of Paramedical Sciences, Kashan University of Medical Sciences, Kashan, Iran
 Department of Radiology, Faculty of Paramedical, Tehran University of Medical Sciences, Tehran, Iran
 Clinical and Anatomical Pathologist at Tehran University of Medical Science, Imam Khomeini Hospital Complex, Tehran, Iran

Clinical and Anatomical Pathologist at Tehran University of Medical Science, Imam Khomeini Hospital Complex, Tehran, Iran
 Department of Molecular Medicine, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran
 Department of Medical Radiation Engineering, Science and Research Branch, Islamic Azad University, Tehran, Iran
 Department of Medical Physics and Biomedical Engineering, Faculty of Medicine, Tehran University of Medical Sciences (International

Campus), Tehran, Iran

7. Department of Physiology, College of Medicine, University of Misan, Misan, Iraq

8. Research Center for Molecular and Cellular Imaging, Tehran University of Medical Sciences (International Campus), Tehran, Iran 9. Radiology and Nuclear Medicine Department, School of Paramedical Sciences, Kermanshah University of Medical Sciences, Kermanshah, Iran

*Corresponding Address: P.O.Box: 6719851351, Radiology and Nuclear Medicine Department, School of Paramedical Sciences, Kermanshah University of Medical Sciences, Kermanshah, Iran Email: najafi_ma@yahoo.com

Received: 10/July/2018, Accepted: 15/September/2018 Abstract

Objective: The Lung is one of the most radiosensitive organs of the body. The infiltration of macrophages and lymphocytes into the lung is mediated via the stimulation of T-helper 2 cytokines such as IL-4 and IL-13, which play a key role in the development of fibrosis. It is likely that these cytokines induce chronic oxidative damage and inflammation through the upregulation of *Duox1* and *Duox2*, which can increase the risk of late effects of ionizing radiation (IR) such as fibrosis and carcinogenesis. In the present study, we aimed to evaluate the possible increase of IL-4 and IL-13 levels, as well as their downstream genes such as *IL4ra1*, *IL13ra2*, *Duox1*, and *Duox2*.

Materials and Methods: In this experimental animal study, male rats were divided into 4 groups: i. Control, ii. Melatonintreated, iii. Radiation, and iv. Melatonin (100 mg/kg) plus radiation. Rats were irradiated with 15 Gy ⁶⁰Co gamma rays and then sacrificed after 67 days. The expressions of *IL4ra1*, *IL13ra2*, *Duox1*, and *Duox2*, as well as the levels of IL-4 and IL-13, were evaluated. The histopathological changes such as the infiltration of inflammatory cells, edema, and fibrosis were also examined. Moreover, the protective effect of melatonin on these parameters was also determined.

Results: Results showed a 1.5-fold increase in the level of IL-4, a 5-fold increase in the expression of *IL4ra1*, and a 3-fold increase in the expressions of *Duox1* and *Duox2*. However, results showed no change for IL-13 and no detectable expression of *IL13ra2*. This was associated with increased infiltration of macrophages, lymphocytes, and mast cells. Melatonin treatment before irradiation completely reversed these changes.

Conclusion: This study has shown the upregulation of *IL-4-IL4ra1-Duox2* signaling pathway following lung irradiation. It is possible that melatonin protects against IR-induced lung injury via the downregulation of this pathway and attenuation of inflammatory cells infiltration.

Keywords: Duox1, Duox2, Lung, Melatonin, Radiation

Cell Journal(Yakhteh), Vol 21, No 3, October-December (Autumn) 2019, Pages: 236-242 ___

Citation: Aliasgharzadeh A, Farhood B, Amini P, Saffar H, Motevaseli E, Rezapoor S, Nouruzi F, Shabeeb Dh, Eleojo Musa A, Mohseni M, Moradi H, Najafi M. Melatonin attenuates upregulation of Duox1 and Duox2 and protects against lung injury following chest irradiation in rats. Cell J. 2019; 21(3): 236-242. doi: 10.22074/cellj.2019.6207.

Introduction

Clinical evidence has shown that more than half of patients with cancers undergo radiotherapy during their course of disease treatment. Normal tissue toxicity is a major limiting factor for tumor control, leading to tumor recurrence and various side effects which affect the quality of life in treated patients (1). Exposure of both normal and tumor cells to ionizing radiation (IR) triggers the production of free radicals such as reactive oxygen species (ROS) and nitric oxide (NO). In addition to direct detrimental effects of IR, these molecules can further amplify damage to cells, resulting in DNA damage and cell death which cause several side effects in the irradiated area (2). The lung is one of the sensitive organs of the human body to the toxic effects of IR. The high radiosensitivity of the lung limits the applied radiation dose for tumor eradication. As non-small cell lung carcinoma (NSCLC) has high resistance to radiotherapy, this results in increased probability of tumor relapses (3). Therefore, to overcome these tumor cells, there is a need for a high dose of IR. However, this may be associated with an increased risk of pneumonitis and fibrosis (4). In recent years, there have been significant improvements in radiotherapy in the delivery of a more precise radiation dose to the tumor volume while sparing normal tissues. However, acute and late normal tissue damage remain an important factor (5). In the lung, radiation-induced side effects such as inflammatory responses (pneumonitis) and fibrosis are the most common limiting factors (6). Currently, there are no appropriate strategies to overcome these complications completely.

Fibrosis is a process resulting from excessive accumulation of collagen due to differentiation of fibroblasts. It is associated with tissue remodeling and affects normal physiological functions (7). Fibrosis and inflammation in some crucial organs such as lung, heart, and gastrointestinal system may threaten patients' life (8). Experimental and clinical studies have shown that abnormal increases in the levels of some cytokines such as TGF- β , IL-1, IL-4, IL-13, TNF- α , etc., have a central role in the development of fibrosis and inflammation (9, 10). The inhibition of some cytokines such as TGF- β and TNF- α has been most widely studied for the amelioration of fibrosis and inflammation (11). In addition to TGF- β , in recent years, some studies have proposed that IL-4 and IL-13 signaling pathways play a key role in the fibrosis process (12). These cytokines trigger the expression of duox1 and duox2 through the upregulation of their cognate receptors on cells, which mediate continuous ROS production and stimulation of fibrosis (13). It has been shown that the upregulation of these cytokines may induce the infiltration of macrophages and maintenance of inflammation (14). As pro-oxidant enzymes such as Duox1, Duox2, NOX1-5, iNOS, and COX-2 play a key role in continuous ROS production and damage to the normal function of organs, suggesting that targeting of these enzymes/genes can help manage normal tissue toxicity during radiotherapy (15).

Treatment with some adjuvants for sensitization of tumor cells or protection of normal tissue cells is one of the most interesting topics in radiation biology. Melatonin is a natural body hormone that regulates circadian rhythm, as well as several mechanisms in the body such as antioxidant enzyme activity (16). In addition, melatonin has a potent interrelationship with immune system cells (17). In response to radiation, melatonin has shown the ability to protect normal tissues through scavenging of free radicals, stimulation of antioxidant enzymes, as well as anti-inflammatory effects (18, 19). Melatonin has also shown an ability to ameliorate radiation or chemotherapy-induced fibrosis in various organs such as the lung, heart, and others (20). In this study, we examined the effect of pre-treatment with melatonin on the development of fibrosis and histopathological damages following irradiation. Also, we evaluated the possible role of melatonin in alleviating increased levels of IL-4 and IL-13, as well as downstream genes such as Duox1 and Duox2 that are involved in late effects of IR.

Materials and Methods

Experimental design

Melatonin was provided (Merck, Germany) and dissolved in 20% ethanol at a concentration of 20 mg/ml. 1 ml of the prepared solution (100 mg/kg) was administered to each rat via intraperitoneal injection (18). For this Interventional-experimental study, twenty healthy adult male Wistar rats (200 ± 20 g) were purchased from the Razi Institute, Tehran, Iran. The procedure of this study was in accordance with the ethical laws for animal

care provided by Kashan University of Medical Sciences, Kashan, Iran. All animals were housed in suitable conditions, including temperature and humidity $(23 \pm 2^{\circ}C \text{ and } 55\%)$, respectively). They were kept under the same light/dark cycle to prevent any effect of light/dark on basal levels of melatonin in different groups (light 8:00 AM to 8:00 PM and dark 8:00 PM to 8:00 AM). Twenty rats were randomly divided into 4 groups (5 rats in each), group 1: control, group 2: melatonin-treated, group 3: radiation, group 4: melatonintreated+radiation. Melatonin was administered orally 30 minutes before irradiation. Irradiation was performed using a ⁶⁰Co source (15 Gy to the whole lung) (21). Sixty-seven days after irradiation, rats were anesthetized, sacrificed, and their lung tissues were extracted. The ventricles were fixed in 10% normal buffer formalin while the auricles were frozen at -80°C for real-time polymerase chain reaction (PCR) and ELISA.

Real-time polymerase chain reaction

Total mRNA was isolated from frozen lung tissue of all groups using TRIzol Reagent (GeneAll, South Korea) while cDNA template was synthesized using cDNA synthesis Kits (GeneAll, South Korea) according to the manufacturer's instructions. *Pgm1* was used as an internal control while the other primers were designed using the Gene runner software and NCBI BLAST tool. Real-time PCR was performed using Corbett's RT PCR (Qiagen, USA). The primer sequences are shown in Table 1. Real-time PCR efficiency for all mentioned genes in Table 1 was determined using the slope of linear regression as described by Pfaffl (22). Five samples in each group were run in duplicates.

Table 1: Forward and reverse primer sequences used in this study Gene Sequence primer (5'-3') IL-4r1 F: GAGTGAGTGGAGTCCCAGCATC R: GCTGAAGTAACAGGTCAGGC IL- 13ra2 F: TCGTGTTAGCGGATGGGGAT R: GCCTGGAAGCCTGGATCTCTA Duox1 F: AAGAAAGGAAGCATCAACACCC R: ACCAGGGCAGTCAGGAAGAT Duox2 F: AGTCTCATTCCTCACCCGGA R: GTAACACACGATGTGGC Pgml F: CATGATTCTGGGCAAGCACG R: GCCAGTTGGGGGTCTCATACAAA

ELISA

The levels of IL-4 and IL-13 cytokines were detected by the IL-4 and IL-13 ELISA kits (Zellbio, Germany) based on the manufacturer's instructions.

Pathological study

Fixed lung tissues were sectioned at 5-micron sections

and then stained with hematoxylin and eosin (H&E) for general tissue characterization. Masson's trichrome staining (MTC) was also performed for the detection of collagen accumulation. The infiltration of mast cells was evaluated using Giemsa staining. All histopathological studies were performed at the Pathology Unit, Imam Khomeini Hospital, Tehran, Iran, with the aid of a light microscope.

Statistical analysis

All statistical analyses were performed using IBM SPSS statistics for windows, version 24.0 (IBM Corp., Armonk, NY, USA). The statistical significance (P<0.05) of mean \pm SD for histopathological and ELISA were analyzed using the ANOVA test followed by post hoc Tukey's HSD. Furthermore, the expression of genes was analyzed using t test.

Results

ELISA

Results showed that irradiation caused a significant increase in the level of IL-4 (702 \pm 102) compared to the control group (469 \pm 89, P<0.05). Treatment with melatonin before irradiation led to a significant decrease in IL-4 (372 \pm 124) compared to the radiation group (P<0.05). Treatment with melatonin alone did not cause any significant change. Results also showed no significant change in the level of IL-13 for all groups (Fig.1).



Fig.1: Results of changes in the levels of IL-4 and IL-13 following irradiation with gamma rays and treatment with melatonin (MLT). **A.** IL-4 and **B.** IL-13. a; Significant compared to control and b; Significant compared to radiation (Rad), ANOVA Tukey's HSD post hoc, P<0.05.

Real-time polymerase chain reaction

Irradiation of lung tissues was associated with a significant elevation in *IL4ra1* compared to the control group (5.21 ± 0.92) folds, P<0.05). When rats were treated with melatonin before exposure to IR, the expression of *ILAra1* was significantly decreased compared to the radiation group (2.60 ± 0.70) folds, P<0.05). Results showed no detectable expression for IL13ra2. The expression of Duox1 gene was elevated following exposure to radiation $(3.18 \pm 0.57 \text{ folds}, P < 0.05)$. When rats were treated with melatonin before exposure to IR, the expression of *Duox1* was significantly attenuated compared to the radiation group $(2.60 \pm 0.70, P < 0.05)$. Results of *Duox2* gene expression showed that irradiation caused an increase in its expression in comparison with the control group (2.95 ± 0.51 folds, P<0.001). Treatment with melatonin led to a significant reduction in Duox2 expression $(1.21 \pm 0.25$ folds compared to control) compared to the radiation group (P<0.01, Fig.2).



Fig.2: The expression of *IL4ra1*, *Duox1* and *Duox2* following irradiation or melatonin treatment before irradiation in lung tissues of rats. **A.** *IL4ra1*, **B.** *Duox1*, and **C.** *Duox2*. a; Significant compared to control and b; Significant compared to radiation (Rad), ANOVA followed by Tukey's HSD post hoc, P<0.05.

Histopathological assay

Histopathological evaluation showed mild fibrosis in the radiation group. However, this reversed completely when melatonin was administered before irradiation. Also, results showed severe infiltration of macrophages and lymphocytes but not neutrophils. Irradiation led to severe alveolar thickening, as well as mild vascular thickening. Results for edema and thrombosis did not show any significant change. Treatment with melatonin could significantly reverse all these changes (Figs.3-5, Table 2).

Table 2: Results of lung irradiation and the protective effect of melatonin				
Variable	Control	Melatonin-treated	Radiation	Radiation+Melatonin
Macrophage infiltration	0.25 ± 50	0.25 ± 50	$2.66\pm0.57^{\rm a}$	$0.80\pm0.83^{\text{b}}$
Lymphocyte infiltration	1.00 ± 0.80	0.50 ± 0.57	$3.00\pm00^{\rm a}$	$0.60\pm0.54^{\rm b}$
Mast cell infiltration	0.00 ± 00	1.00 ± 50	$4.00\pm00^{\rm a}$	3.50 ± 0.50
Neutrophil infiltration	0.50 ± 0.57	0.50 ± 0.57	0.00 ± 00	0.60 ± 0.54
Alveolar thickness	0.25 ± 50	0.25 ± 50	$2.00\pm1.00^{\rm a}$	$0.20\pm0.44^{\rm b}$
Vascular thickness	0.00 ± 00	0.00 ± 00	$1.00\pm00^{\rm a}$	$0.00\pm00^{\rm b}$
Edema and thrombosis	0.00 ± 00	0.00 ± 00	1.00 ± 0.57	0.00 ± 00
Fibrosis	Absent	Absent	Mild	Absent

Results were scored from 0-3 as 0; Normal, 1; Mild, 2; Severe, 3; Very severe, ^a; Significant compared to control group, and ^b; Significant compared to radiation group. Data are presented as mean ± SD.



Fig.3: Histopathological investigation of the protective effect of melatonin on radiation-induced lung injury. Control and melatonin groups: no infiltration of macrophages and lymphocytes, as well as normal vascular and alveolar thickening, radiation: severe infiltration of macrophages and lymphocytes, as well as vascular thickening, while alveolar thickening mildly changed. **A.** Control; **B.** Melatonin, **C.** Radiation, **D.** Radiation+Melatonin (H&E staining ×100).



Fig.4: Results of trichrome staining showed a mild collagen deposition, while treatment with melatonin completely reversed collagen deposition. A. Control, B. Melatonin, C. Radiation, and D. Radiation+Melatonin (Masson's Trichrome staining ×100).



Fig.5: Infiltration of mast cells following irradiation of lung tissues in rats. The administration of melatonin before irradiation could not significantly attenuate mast cell infiltration. A. Control, B. Melatonin, C. Radiation, and D. Radiation+Melatonin (Giemsa staining ×100).

Discussion

The aim of our study was to evaluate changes in the levels of two important pro-fibrotic cytokines and downstream pro-oxidant genes such as *Duox1* and *Duox2*. Moreover, we detected the possible modulatory effect of melatonin on the changes in the level of these factors. In the present study, we revealed that irradiation of rats' chest led to a significant increase in the expression of *IL4ra1*, *Duox1*, and *Duox2* genes. ELISA results showed that the level of IL-4 was increased significantly.

In contrast to IL-4, results suggest that irradiation of lung tissue did not cause any significant change in the level of IL-13. Moreover, real-time PCR results showed no detectable expression of IL13ra2. Probably, the upregulation of *Duox1* was mediated by other signaling pathways, not by IL13ra2. A study has shown that, in addition to IL-13, *Duox1* can be upregulated through IL-4 (23). There is a possibility that IL-4 upregulates both Duox1 and Duox2 gene expressions through the stimulation of IL4ra1, while IL-13 is not involved in this pathway. There is also a possibility of the involvement of other cytokines such as IFN- γ (24, 25). Our results are in agreement with a study by Groves et al. (26) which showed that IL-4 is involved in the maintenance of macrophages and lung injury following irradiation. However, this study proposed that the development of fibrosis may be induced by other immune mediators but not IL-4. By contrast to our results, Chung et al. (14) reported that after exposure to IR, increased the level of IL-13 but not IL-4 was responsible for the development of lung injuries such as macrophage infiltration and fibrosis. They showed that IL-13 deficiency could reverse lung injury and reduce the expression of genes involved in fibrosis such as TGF- β , matrix metalloproteinase-2 (MMP-2), and MMP-3. However, this study differed from ours in the sense that they used wild-type c57BL/6NcR mice and a longer time of evaluation.

As earlier mentioned, the lung is one of the most critical organs for the detrimental effects of IR. It has been reported that the long-term exposure of the lung to radiotherapy due to cancer therapy or iodine therapy for thyroid cancer with metastasis can cause death via pneumonitis or fibrosis (27). In addition to clinical importance for cancer therapy, lung injury may appear following accidental exposure to IR. In this situation, lung late effects may appear following non-uniform whole body exposure or inhalation of radionuclides (28). As the development of lung injury may take a long time to appear, a knowledge of the mechanisms involved in radiation-induced pneumonitis and fibrosis can help better management of them. Although most studies have detected the elevated level of TGF- β associated with radiation fibrosis, some studies suggest the greater importance of IL-4. It has been proposed that IL-4 plays a central role via the stimulation of other pro-inflammatory and pro-fibrotic cytokines (10). Infiltration of inflammatory cells including macrophages and lymphocytes in irradiated tissues is the source of increased release of IL-4. The accumulation

of macrophages and lymphocytes and elevated levels of inflammatory cytokines promote ROS and NO through the stimulation of reduction/oxidation interactions (29). Increased oxidative injury induces a higher degree of inflammation and fibrosis in a positive feedback loop that could finally lead to death (28).

Histopathological evaluation showed that irradiation of the lung led to severe infiltration of mast cells, macrophages, and lymphocytes, but not neutrophils. This suggests that neutrophil infiltration is involved in late effects of lung injury by IR. Moreover, the histological findings showed mild fibrosis, alveolar, and vascular thickening. Except for mast cell infiltration, all other pathological changes following exposure to IR were alleviated when melatonin was administered 30 minutes before irradiation. Also, melatonin could blunt the upregulation of IL-4 and downstream signaling in IL4ra1 and Duox2. Since macrophages are the main source for the secretion of IL-4 during pneumonitis or fibrosis, it seems that the upregulation of IL-4 signaling after irradiation, as well as the downregulation of that in response to melatonin pretreatment prior to irradiation, is responsible for modulating the infiltration of macrophages. Regarding *IL4ra1* and *Duox2* can promote continuous ROS production, it is possible that IL-4 signaling plays a key role in radiation-induced fibrosis and vascular injury in the lung. Melatonin treatment before irradiation could reverse the upregulation of these genes completely.

Previous studies have shown that melatonin has the ability to reduce radiation injury via the modulation of several signaling pathways. Melatonin is an FDAapproved drug which has a peak time of absorption up to 40 minutes and a half-life of 1-2 hours (30, 31). Therefore, its administration between 30-60 minutes before exposure to radiation is a common method for radiobiological studies. Melatonin has shown potent anti-inflammatory effects via the prevention of radiation-induced DNA damage and cell death (32). Melatonin can also elevate DNA repair capacity to mitigate cell death (19). By means of the inhibition of Toll-like receptors (TLRs), transcription factors, pro-oxidant enzymes, as well as pro-fibrotic and pro-inflammatory cytokines melatonin attenuates redox activity and relieves late effects of IR (20, 33). As a result of these properties, melatonin is appropriate for radiation countermeasure, protection, and mitigation of lung injury by IR (34).

Conclusion

Results of this study showed that irradiation of rats' lung led to a significant increase in the level of IL-4 and prooxidant genes such as *Duox1* and *Duox2*. However, we did not observe any significant increase in the level of IL-13, as well as the expression of *IL13ra2*. This could be an indication that radiation induces lung inflammation and fibrosis via the upregulation of IL-4 but not IL-13. This suggests that the infiltration of macrophages plays a key role in the stimulation of IL-4 and its downstream genes. In addition, we showed that melatonin administration before irradiation reversed the infiltration of macrophages and lymphocytes, as well as the upregulation of IL-4, *IL4ra1*, *Duox1*, and *Duox2*.

Acknowledgments

This study was financially supported by Kashan University of Medical Sciences. There is no conflict of interest in this study.

Authors' Contributions

A.A., E.M., M.M., H.M., M.N.; Were involved in study design, manuscript preparation, and editing. D.S., A.E.M.; Were involved in animal work, preparing first draft and edit for grammar. H.S.; Provided pathology results. B.F., P.A., S.R., F.N.; Were involved in animal work, radiotherapy, RT-PCR, and ELISA. All authors read and approved the final manuscript.

References

- Yahyapour R, Shabeeb D, Cheki M, Musa AE, Farhood B, Rezaeyan A, et al. Radiation protection and mitigation by natural antioxidants and flavonoids; implications to radiotherapy and radiation disasters. Curr Mol Pharmacol. 2018 (ahead of print).
- Mortezaee K, NH Goradel, P Amini, D Shabeeb, A E Musa, M Najafi. NADPH oxidase as a target for modulation of radiation response; implications to carcinogenesis and radiotherapy. Curr Mol Pharmacol. 2018 (ahead of print).
- 3. Abratt RP, Morgan GW. Lung toxicity following chest irradiation in patients with lung cancer. Lung Cancer. 2002; 35(2): 103-109.
- Ghafoori P, Marks LB, Vujaskovic Z, Kelsey CR. Radiation-induced lung injury. Assessment, management, and prevention. Oncology. 2008; 22(1): 37-47; discussion 52-53.
- Tsoutsou PG, Koukourakis MI. Radiation pneumonitis and fibrosis: mechanisms underlying its pathogenesis and implications for future research. Int J Radiat Oncol Biol Phys. 2006; 66(5): 1281-1293.
- Haddadi GH, Rezaeyan A, Mosleh-Shirazi MA, Hosseinzadeh M, Fardid R, Najafi M, et al. Hesperidin as radioprotector against radiation-induced lung damage in rat: a histopathological study. J Med Phys. 2017; 42(1): 25-32.
- Zhao W, Robbins ME. Inflammation and chronic oxidative stress in radiation-induced late normal tissue injury: therapeutic implications. Curr Med Chem. 2009; 16(2): 130-143.
- Bahig H, Filion E, Vu T, Chalaoui J, Lambert L, Roberge D, et al. Severe radiation pneumonitis after lung stereotactic ablative radiation therapy in patients with interstitial lung disease. Pract Radiat Oncol. 2016; 6(5): 367-374.
- Chen Y, Williams J, Ding I, Hernady E, Liu W, Smudzin T, et al. Radiation pneumonitis and early circulatory cytokine markers. Semin Radiat Oncol. 2002; 12(1 Suppl 1): 26-33.
- Di Maggio FM, Minafra L, Forte GI, Cammarata FP, Lio D, Messa C, et al. Portrait of inflammatory response to ionizing radiation treatment. J Inflamm (Lond). 2015; 12: 14.
- Rube CE, Uthe D, Schmid KW, Richter KD, Wessel J, Schuck A, et al. Dose-dependent induction of transforming growth factor beta (TGF-beta) in the lung tissue of fibrosis-prone mice after thoracic irradiation. Int J Radiat Oncol Biol Phys. 2000; 47(4): 1033-1042.
- Jakubzick C, Kunkel SL, Puri RK, Hogaboam CM. Therapeutic targeting of IL-4- and IL-13-responsive cells in pulmonary fibrosis. Immunol Res. 2004; 30(3): 339-349.
- Wu Y, Doroshow JH. IL-4/IL-13 induce Duox2/DuoxA2 expression and reactive oxygen production in human pancreatic and colon cancer cells. Cancer Res. 2014; 74(19 Suppl): 5-9.
- 14. Chung SI, Horton JA, Ramalingam TR, White AO, Chung EJ, Hudak KE, et al. IL-13 is a therapeutic target in radiation lung injury. Sci Rep. 2016; 6: 39714.

- Cheki M, Yahyapour R, Farhood B, Rezaeyan A, Shabeeb D, Amini P, et al. COX-2 in radiotherapy: a potential target for radioprotection and radiosensitization. Curr Mol Pharmacol. 2018; 11(3): 173-183.
- Tripathi DN, Jena GB. Effect of melatonin on the expression of Nrf2 and NF-kappaB during cyclophosphamide-induced urinary bladder injury in rat. J Pineal Res. 2010; 48(4): 324-331.
- Miller SC, Pandi-Perumal SR, Esquifino AI, Cardinali DP, Maestroni GJ. The role of melatonin in immuno-enhancement: potential application in cancer. Int J Exp Pathol. 2006; 87(2): 81-87.
- Najafi M, Salehi E, Farhood B, Nashtaei MS, Hashemi Goradel N, Khanlarkhani N, et al. Adjuvant chemotherapy with melatonin for targeting human cancers: a review. J Cell Physiol. 2019 (ahead of print).
- Farhood B, Goradel NH, Mortezaee K, Khanlarkhani N, Salehi E, Nashtaei MS, et al. Melatonin as an adjuvant in radiotherapy for radioprotection and radiosensitization. Clin Transl Oncol. 2018 (ahead of print).
- Farhood B, Goradel NH, Mortezaee K, Khanlarkhani N, Najafi M, Sahebkar A. Melatonin and cancer: From the promotion of genomic stability to use in cancer treatment. J Cell Physiol. 2018 (ahead of print).
- Ghosh SN, Zhang R, Fish BL, Semenenko VA, Li XA, Moulder JE, et al. Renin-Angiotensin system suppression mitigates experimental radiation pneumonitis. Int J Radiat Oncol Biol Phys. 2009; 75(5): 1528-1536.
- 22. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001; 29(9): e45.
- Eskalli Z, Achouri Y, Hahn S, Many MC, Craps J, Refetoff S, et al. Overexpression of interleukin-4 in the thyroid of transgenic mice upregulates the expression of Duox1 and the anion transporter pendrin. Thyroid. 2016; 26(10): 1499-1512.
- Ameziane-Él-Hassani R, Talbot M, de Souza Dos Santos MC, Al Ghuzlan A, Hartl D, Bidart JM, et al. NADPH oxidase DUOX1 promotes long-term persistence of oxidative stress after an exposure to irradiation. Proc Natl Acad Sci USA. 2015; 112(16): 5051-5056.
- 25. Harper RW, Xu C, Eiserich JP, Chen Y, Kao CY, Thai P, et al. Differential regulation of dual NADPH oxidases/peroxidases, Duox1 and Duox2, by Th1 and Th2 cytokines in respiratory tract epithelium. FEBS Lett. 2005; 579(21): 4911-4917.
- Groves AM, Johnston CJ, Misra RS, Williams JP, Finkelstein JN. Effects of IL-4 on pulmonary fibrosis and the accumulation and phenotype of macrophage subpopulations following thoracic irradiation. Int J Radiat Biol. 2016; 92(12): 754-765.
- Hebestreit H, Biko J, Drozd V, Demidchik Y, Burkhardt A, Trusen A, et al. Pulmonary fibrosis in youth treated with radioiodine for juvenile thyroid cancer and lung metastases after chernobyl. Eur J Nucl Med Mol Imaging. 2011; 38(9): 1683-1690.
- Yahyapour R, Motevaseli E, Rezaeyan A, Abdollahi H, Farhood B, Cheki M, et al. Reduction-oxidation (redox) system in radiationinduced normal tissue injury: molecular mechanisms and implications in radiation therapeutics. Clin Transl Oncol. 2018; 20(8): 975-988.
- Yahyapour R, Motevaseli E, Rezaeyan A, Abdollahi H, Farhood B, Cheki M, et al. Mechanisms of radiation bystander and nontargeted effects: implications to radiation carcinogenesis and radiotherapy. Curr Radiopharm. 2018; 11(1): 34-45.
- Andersen LP, Werner MU, Rosenkilde MM, Harpsøe NG, Fuglsang H, Rosenberg J, et al. Pharmacokinetics of oral and intravenous melatonin in healthy volunteers. BMC Pharmacol Toxicol. 2016; 17: 8.
- Gooneratne NS, Edwards AY, Zhou C, Cuellar N, Grandner MA, Barrett JS. Melatonin pharmacokinetics following two different oral surge-sustained release doses in older adults. J Pineal Res. 2012; 52(4): 437-445.
- Jou MJ, Peng TI, Yu PZ, Jou SB, Reiter RJ, Chen JY, et al. Melatonin protects against common deletion of mitochondrial DNA-augmented mitochondrial oxidative stress and apoptosis. J Pineal Res. 2007; 43(4): 389-403.
- Talib WH. Melatonin and cancer hallmarks. Molecules. 2018; 23(3). pii: E518.
- Yahyapour R, Amini P, Rezapoor S, Rezaeyan A, Farhood B, Cheki M, et al. Targeting of inflammation for radiation protection and mitigation. Curr Mol Pharmacol. 2018; 11(3): 203-210.

Preliminary Findings of Platelet-Rich Plasma-Induced Ameliorative Effect on Polycystic Ovarian Syndrome

Samira Seyyed Anvari, M.Sc.¹, Gholamreza Dehgan, Ph.D.², Mazdak Razi, Ph.D.^{3*}

1. Department of Biology, Collage of Post Graduate, Ahar Islamic Azad University, Ahar, Iran 2. Department of Biochemistry, Faculty of Natural Science, University of Tabriz, Tabriz, Iran 3. Department of Basic Science, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran

*Corresponding Address: P.O.Box: 1177, Department of Basic Science, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran Email: mazdak.razi@gmail.com

Received: 4/April/2018, Accepted: 19/November/2018

Abstract -

Objective: Polycystic ovarian syndrome (PCOS) is characterized by hormonal imbalance, oxidative stress and chronic anovulation. The present study was designed to assess ameliorative effect of auto-locating platelet-rich plasma (PRP), as a novel method, for inhibiting PCOS-induced pathogenesis in experimentally-induced hyperandrogenic PCOS.

Materials and Methods: In this experimental study, 30 immature (21 days old) female rats were assigned into five groups, including control (sampled after 30 days with no treatment), 15 and 30 days PCOS-sole-induced as well as 15 and 30 days PRP auto-located PCOS-induced groups. Serum levels of estrogen, progesterone, androstenedione, testosterone, follicle stimulating hormone (FSH), luteinizing hormone (LH), ovarian total antioxidant capacity (TAC), malondialdehyde (MDA), glutathione peroxidase (GSH-px) and superoxide dismutase (SOD) were evaluated. Expression of estrogen receptor α (*Era*), β (*Erβ*) and *c-Myc* were assessed. Finally, the numbers of intact follicles per ovary and mRNA damage ratio were analyzed.

Results: PRP groups significantly (P<0.05) decreased serum levels of FSH, LH, testosterone and androstenedione and remarkably (P<0.05) increased estrogen and progesterone syntheses versus PCOS-sole groups. The PRP auto-located animals exhibited increased TAC, GSH-px and SOD levels, while they showed diminished MDA content (P<0.05) versus PCOS-sole groups. The PRP auto-located groups exhibited an elevated expression of $Er\alpha$ and $Er\beta$ versus PCOS-sole groups. Moreover, PRP groups significantly (P<0.05) decreased *c-Myc* expression and mRNA damage compared to PCOS-sole groups, and remarkably improved follicular growth.

Conclusion: PRP is able to regulate hormonal interaction, improve the ovarian antioxidant potential as well as folliculogenesis and its auto-location could be considered as a novel method to prevent/ameliorate PCOS-induced pathogenesis.

Keywords: Folliculogenesis, Oxidative Stress, Platelet-Rich Plasma, Polycystic Ovarian Syndrome, Rat

Cell Journal(Yakhteh), Vol 21, No 3, October-December (Autumn) 2019, Pages: 243-252 _

Citation: Seyyed Anvari S, Dehgan Gh, Razi M. Preliminary findings of platelet-rich plasma-induced ameliorative effect on polycystic ovarian syndrome. Cell J. 2019; 21(3): 243-252. doi: 10.22074/cellj.2019.5952.

Introduction

Polycystic ovarian syndrome (PCOS) is an exceptionally common disorder, which is widely observed in premenopausal women. It is characterized by an increased serum level of androgens (hyperandrogenism), chronic anovulation and presence of the polycystic ovarian morphology (1). According to the Rotterdam consensus in 2003, chronic anovulation or oligomenorrhea, clinical or biochemical hyperandrogenism, and polycystic ovarian morphology are declared as main criteria for PCOS (2). Among the different mentioned phenotypes, ovarian hyperandrogenism has gained higher attenuations. Indeed, in PCOS, an intrinsic steroidogenic defect of theca cells results in ovarian hyperandrogenism. Accordingly, increased LH and enhanced insulin levels amplify inherent impairment of steroidogenesis in theca cells (3). In addition to hyperandrogenism symptoms, follicle stimulating (FSH) and luteinizing (LH) hormones upregulation, as well as estrogen and progesterone reduction levels have been reported in PCOS patients (3, 4). Estrogen interacts with two distinct estrogen receptors (ERs), namely ER α and ER β (5), both of which regulate variety of genes expression, leading to cellular proliferation and differentiation in both male and female gonads (6). In rodents, $Er\alpha$ is expressed exclusively in theca cells, whereas $Er\beta$ is expressed especially in granulosa cells (GCs) (7). Several evidences, including failed follicular maturation, anovulation and hemorrhagic cysts formation are reported for $Er\alpha$ knockout (α ERKO) mice ovaries (8, 9). The $Er\beta$ -related phenotypes are partially different from those related to $Er\alpha$. Actually, $Er\beta$ knockout (β ERKO) mice ovaries appear normal, exhibiting follicles at all stages of development. Meanwhile, these mice represent fewer corpora lutea, resulting in mild subfertility problems. Moreover, failed response to exogenous gonadotropins as well as a severe deficiency in response to the LH/human chorionic gonadotropin (hCG) ovulatory stimulus have been reported in β ERKO mice ovaries (5).

In addition to estrogen and ERs. the proto-oncogene cellular myc (c -Myc), as a transcription factor, participates in cellular proliferation pathway (10). Although c-Myc protein has been illustrated to induce both growth and oncogenic properties, very early studies have shown its pro-apoptotic characteristic in ovarian tissue. c-Myc is expressed in GCs at all stages of follicular development and in oocyte of primordial follicles, suggesting its role in remodeling the

ovarian local tissue following atresia and luteolysis (11). Meanwhile, the massive expression of c-Myc protein in GCs and theca interna of atretic follicles, as well as peripheral theca lutein cells implies the pro-apoptotic characteristic of c-Myc in ovaries (12).

According to the previous reports, PCOS is frequently associated with oxidative stress. Various investigations have shown remarkable enhancement in circulating malondialdehyde (MDA) as well as significant reduction in serum superoxide dismutase (SOD), and glutathione peroxidase (GSH-px) of patients with PCOS (13, 14). Indeed, there is a positive correlation between obesity, insulin resistance, hyperandrogenemia, chronic inflammation and oxidative stress in PCOS ovaries (15). Therefore, the impressively-induced oxidative stress is considered as a potential inducer of PCOS-related pathogenesis (13).

Platelet-rich plasma (PRP) or autologous platelet gel, has gained high attentions in musculoskeletal medicine, hemostasis and wound healing, inhibiting immune reactions, aesthetic plastic surgery (16), spinal fusion (17) and heart bypass surgery (18), in addition to treatment of chronic skin and soft-tissue ulcers (19). α -granules of platelets are comprised of numerous proteins, including platelet-derived growth factor (PDGF), transforming growth factor (TGF)- β , platelet factor 4 (PF4), interleukin (IL)-1, platelet-derived angiogenesis factor (PDAF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), platelet-derived endothelial growth factor (PDEGF), epithelial cell growth factor (ECGF) insulin-like growth factor (IGF), osteocalcin, osteonectin, fibrinogen, vitronectin, fibronectin, and thrombospondin (TSP)-1 (16). Further studies have shown promoting effect of PRP in different therapeutic innervations (20).

Minding the essential role of growth hormones in both early and late folliculogenesis and in initiating oocyte growth, as well as cell proliferation and inhibiting apoptosis (especially at later stages of development), this question arises what the possible effect of PRPrelated growth factors on different molecular elements is, in PCOS-induced ovaries. Therefore, here we aimed to uncover the possible ameliorative effect of PRP on hyperandrogenic PCOS-induced derangements in ovarian tissue. The possible PRP-related ameliorative effects were assessed in five well-established categories, including: i. Alterations at gonadotropins, androgens, estrogen and progesterone levels, ii. Changes in expression of Era and Er β (as important receptors participating in folliculogenesis), iii. Alteration in c-Myc expression (as important proto-oncogene involved in cell proliferation/ apoptosis), iv. Ovarian antioxidant status, and finally v. Follicular atresia and/or growth ratio.

Materials and Methods

Chemicals and materials

Specific commercial kits were purchased for analysis of rat testosterone (Mybiosource, USA), androstenedione

(Mybiosource, USA), estrogen (Bio Vender, Czech Republic), progesterone (Crystal Chem, USA), LH (Mybiosource, USA), FSH (Bio Vender, Japan). Primary antibodies were provided for $\text{Er}\alpha$, $\text{Er}\beta$ and c-Myc (Rabbit-Antimouse $\text{Er}\alpha$, $\text{Er}\beta$ and c-Myc; Biocare, USA). Commercial kits for SOD and GSH-px were obtained from RANDOX reagents company (Germany). All other chemical agents were commercial products of analytical grade.

Animals, PCOS induction and experimental design

The current experimental study was performed on animal models. To conduct it, 30 immature (21 days old) female Sprague-Dawley rats were assigned into five groups (six rats in each group), including control (sampled after 30 days), PCOS-induced (sampled 15 and 30 days of post PCOS induction) and PRP auto-located PCOSinduced (sampled 15 and 30 days of post PCOS induction) groups. The animals were given ad libitum access to food and water, kept at room temperature (21-23°C) on a 12:12 light:dark cycle. The hyperandrogenic PCOSlike condition was induced based on the previous study by Honnma et al. (21). Briefly, dehydroepiandrosterone (DHEA, 6 mg/100 g body weight/0.2 ml sesame oil) was subcutaneously injected to 22 days old rats, every evening for 15 days. The animals in the control group were received 0.2 ml sesame oil every evening for the corresponding length of time. Extra cares were taken and no inflammatory reaction was observed at the injected site, during the trial (Fig.1). All experimental protocols were approved and monitored by the Ethical Committee in Animal Experimentation of Urmia University (Urmia, Iran).

Platelet-rich plasma preparation, activation and count

To perform the experimental procedures and PRP preparation, the animals were anesthetized through intraperitoneal injection of xylazine (6 mg/kg, Trittau, Germany) and ketamine (70 mg/kg, Alfason-Woerden, Netherland). Next, the cannulation of caudal vena cava was submitted. 5 ml disposable syringe containing 0.35 ml of 10% sodium citrate was used to collect 3.15 ml PRP of each animal. The blood samples were kept in 5 ml sterile silicone vacuum tubes. In order to replace the same amount of blood, sterile saline was immediately injected. PRP preparation was carried out based on the proposed protocol by Messora et al. (22). Briefly, the collected blood samples were firstly centrifuged (Beckman J-6M, UK) at 160 rpm, 22°C for 20 minutes. Then, red blood cell component (lower fraction) and serum component, as an upper straw-yellow turbid fraction, were observed. Thereafter, a point was marked at 1.4 mm below the line dividing two fractions. All contents above the marked point were pipetted and transferred to another 5 ml vacuum tube. The sample was then centrifuged at 400 rpm, for 15 minutes, resulting in two components, including plateletpoor plasma (PPP) and PRP in the bellow part (Fig.1A, B). Next, similar amounts of PRP and PPP (0.35 ml) were pipetted and transferred to different sterile dappen

dishes. After that, they were activated by adding 0.05 ml of 10% calcium chloride solution to each 1 ml of PRP or PPP. Finally, the platelets were manually counted (8.08 \pm 3.24×10⁶/µl) using the Neubauer chamber, through Olympus optical microscope (CH-2, Japan), at ×40 magnification objective lens.

Auto-location of platelet-rich plasma

Following PCOS induction, PRPs were collected and activated as previously described and subsequently $1.00 \times 10^{6}/\mu$ l PRPs were auto-located from each animal into the mesovarian enclosed to ovaries (Fig.1C).

Histological analyses

At the end of experiment, light anesthesia was induced to animals using 5% ketamine (40 mg/kg) in addition to 2% xylazine (5 mg/kg), intraperitoneally and then euthanized by especial CO, device (ADACO, Iran). Next, the ovarian tissues were dissected out and fixed in 10% formalin for 72 hours. Routine sample processing was performed using ascending alcohol and the samples were then embedded in paraffin. Thereafter, serial sections were prepared by rotary microtome (Leitz Wetzlar, Germany) and stained with hematoxylin-eosin. To perform histomorphometric analyses, follicles were classified to preantral and antral types. Follicles with intact/complete layers of GCs and theca cells, ordinary cytoplasm of oocyte and intact nuclei were considered as normal/intact follicles. Follicles with GC dissociation, early antrum formation, luteinized elongated GCs were considered as atretic types. The atretic preantral and antral follicles were counted in serial sections for each sample and compared between groups.

Analyses of RNA damage

Darzynkiewicz method was considered to assess the

RNA damage (23). In brief, ether alcohol was used to wash the ovaries and thereafter, 10 µm sections were obtained using cryostat microtome (Huntingdom, UK). Different degrees of ethanol were used to fix the sections. Next, the sections were rinsed in acetic acid (1%) and washed in distilled water. The slides were stained in acridine-orange (3-5 minutes) and then counterstained in phosphate buffer (pH=6.85, 2 minutes). Finally, the fluorescent colors differentiation was induced by calcium chloride. The follicular cells with RNA damage were characterized with loss and/or faint red stained RNA. The normal cells were marked with bright red fluorescent RNA.

Immunohistochemical staining

Tissue slides were heated at 60°C (25 minutes) in a hot-air oven (Venticell, Germany). Tissue sections were then dewaxed in xylene (2 changes, each change 5 minutes) and rehydrated. Following antigen retrieval process (in 10 mM sodium citrate buffer), the immunohistochemical (IHC) staining was conducted based on the manufacturer's protocol (Biocare, USA). Briefly, endogenous peroxidases were blocked by 0.03% hydrogen peroxide containing sodium acid. The sections were washed gently and thereafter, incubated with Era (1:500), Er β (1:600) and c-Myc (1:500) biotinylated primary antibodies in 4°C, overnight. The slides were then rinsed gently with phosphate-buffered saline (PBS) and placed in a humidified chamber with a sufficient amount of streptavidin conjugated to horseradish peroxidase in PBS, containing an antimicrobial agent, for 15 minutes. Next, DAB chromogen was used to mark target proteins. Counterstaining was conducted by hematoxylin. Finally, the sections were dipped in ammonia (0.037 ml), rinsed in distilled water and coverslipped. The positive immunohistochemical reaction was visualized as brown.



Fig.1: Summarized schematic diagram for animals platelet-rich plasma (PRP) preparation, activation, auto-location and animal grouping of the study. A. Blood sampling from caudal vena cava, B. PRP preparation, and C. PRP auto-location.

RNA isolation, cDNA synthesis and reverse transcription-polymerase chain reaction

Previously collected and stored (-70°C) ovaries were used for total RNA extraction, based on the standard TRIZOL method (24). In brief, 20-30 mg of ovarian tissue from individual animal of each group was homogenized in 1 ml of TRIZOL (Thermo Fisher Scientific, USA) and the colorless aqueous phase was collected. The extra care was taken in order to avoid genomic DNA contamination. The amount of total RNA was determined using nanodrop spectrophotometer (260 nm and A260/280 ratio=1.8-2.0), and thereafter the samples were stored at -70°C. For reverse transcription-polymerase chain reaction (RT-PCR), cDNA was synthesized in a 20 µl reaction mixture containing 1 µg total RNA, oligo (dT) primer (1 µl), 5×reaction buffer (4 μ l), RNase inhibitor (1 μ l), 10 mM dNTP mix (2 µl), M-MuLV Reverse Transcriptase (1 µl) according to the manufacturer's protocol (Fermentas, Germany). Cycling protocol for 20 µl reaction mix was performed for 5 minutes at 65°C, followed by 60 minutes at 42°C, and 5 minutes at 70°C to terminate the reaction. PCR reaction was carried out in a total volume of 27 µl containing PCR master mix (13 µl), FWD and REV specific primers (each 1 μ l), and cDNA as a template (1.5 μ l) and nuclease free water (10.5 μ l). The PCR conditions were run as follows: one cycle of general denaturation at 95°C for 3 minutes, followed by 35 cycles of 95°C for 20 seconds, annealing temperature (50°C for c-Myc, 62°C for $Er\alpha$, 58°C for $Er\beta$ and finally 60°C for β -Actin) for 60 seconds and elongation at 72°C for 1 minute, before terminating cycle at 72°C for 5 minutes (25, 26). Specific primers were designed and manufactured by Cinna-Gen company (Iran). Primers pair sequences, for individual genes are presented in Table 1.

Determination of ovarian TAC, MDA, SOD and GSHpx contents

In order to analyze ovarian antioxidant capacity, the tissues were washed three times with 0.9% NaCl solution, and using Teflon-end-on homogenizator (Elvenjempotter,

USA), each ovary tissue was homogenized in 9 ml of 1.15% KCl. Thereafter, the homogenates were centrifuged at 4000 rpm. MDA content was next measured based on the thiobarbituric acid (TBA) reaction and the sample absorbance ratios were measured and recorded at 532 nm (27). Ovarian activities of SOD and GSH-px were analyzed using the commercial measurement kits of RAN-SOD and RAN-SOL (Rodex, Germany) and the absorbance ratio of samples were measured and recorded at 340 nm. Ovarian TAC status was also evaluated based on the ferric reducing antioxidant power (FRAP) assay and the absorbance of samples was measured and recorded at 593 nm (28). Finally, the ovarian protein contents were evaluated based on the Lowry method (29).

Serum sampling and hormonal analyses

Blood sample of each animal was collected directly from heart and serum was separated by centrifugation (3000 rpm for 5 minutes). Finally, serum progesterone, estrogen, testosterone, androstenedione, FSH and LH concentrations were measured. Serum levels of the hormones were evaluated by ELISA method. Moreover, intra- and inter-assay coefficient variances of the current experiment were respectively estimated as 3.1, 3.9, 4.2, 3.2 and 4.6% for testosterone, estrogen, androstenedione, LH and FSH (for 10 times), as well as 7.9, 6.3, 6.7, 7.2 and 6.3% for testosterone, estrogen, androstenedione, LH and FSH (for 10 times).

Statistical analysis and imaging

The data were analyzed using SPSS for windows, version 16.0 (SPSS Inc., Chicago, IL, USA), presented as mean \pm SD and the comparison between groups were made by analysis of variance (ANOVA) followed by Bonferroni post-hoc test. Finally, the value of P<0.05 was considered significant. SONY onboard camera (Zeiss, Cyber-Shot, Japan) was used to take photomicrographs. The pixel-based frequency for mRNA damage was analyzed using Image pro-insight software (Version 9:00, USA).

Table 1: Nucleotide sequences and products size of the primers used in RT-PCR			
Target genes	Primer sequence (5´-3´)	AT (°C)	Product size (bp)
Erα	F: CCGGTCTATGGCCAGTCGAGCATC	62	380
	R: GTAGAAGGCGGGAGGGCCGGTGTC		
Erβ	F: AGCGACCCATTGCCAATCA	58	290
	R: CTGGCACAACTGCTCCCACTAA		
с-Мус	F: AACTTACAATCTGCGAGCCA	50	420
	R: AGCAGCTCGAATTTCTTCCAGATAT		
B-Actin	F: GTTACCAGGGCTGCCTTCTC	60	310
	R: GGGTTTCCCGTTGATGACC		

RT-PCR; Reverse transcription-polymerase chain reaction and AT; Annealing temperature.

Results

Platelet-rich plasma diminished PCOS-induced follicular atresia and mRNA damage

Animals of the PCOS-sole groups exhibited pie size atretic/cystic follicles in the cortex of ovaries. However, the animals of PRP auto-located groups showed corpus luteom formation, representing physiologic ovulation. Histological observations showed that PRP decreased PCOS-induced follicular atresia. Accordingly, the animals of PRP auto-located groups exhibited remarkably (P<0.05) higher number of intact preantral and antral follicles/ovary versus non-treated PCOS-induced animals. Moreover, special fluorescent staining was done to assess PCOS-induced mRNA damage. The animals in PCOS-sole groups showed intensive mRNA damage. Meanwhile, those of PRP auto-located groups exhibited diminished mRNA damage in pixel based frequency analyses. No histopathological change was seen in the control group (Fig.2A-D).



Fig.2: Cross sections from ovarian tissue and mRNA damage. **A.** Hematoxylin and eosin staining of ovarian cross sections in different groups; see massive cystic (CF) and atretic follicles distribution on the ovaries of the PCOS-sole groups. The ovaries from PRP-treated groups represent corpora lutea (CL) on the ovaries following 15 and 30 days, **B.** Fluorescent staining for RNA damage: the cross sections of PCOS-sole groups represent damaged RNA in yellowish and/or green fluorescent spots (arrows). Meanwhile, the sections of PRP-treated groups exhibit intact RNA in bright red fluorescent reactions (arrows), **C.** Pixel based frequency assay for bright red fluorescent reactivity (marking intact RNA content) in 209×10 µm of tissue; see diminished reactivity in the PCOS-sole groups, and **D.** Mean ± SD of intact preantral and antral follicles in different groups. Different letters represent significant differences (P<0.05) between groups (n=6). PCOS; Polycystic ovarian syndrome, PRP; Platelet-rich plasm, Pre-antral: a vs. b, d; e; P=0.001, a vs. c; P=0.01, b vs. c; P=0.02, b vs. e; P=0.02, c vs. d; P=0.001, c vs. e; P=0.02, Antral: a vs. b, d; P=0.001, b vs. c; P=0.001, b vs. d; P=0.01 (scale bar: 300 µm).

Platelet-rich plasma enhanced $Er\alpha$ and $Er\beta$ expression

The animals of PCOS-sole groups exhibited diminished mRNA levels of $Er\alpha$ and $Er\beta$ compared to the control group. However, semi-quantitative RT-PCR analyses exhibited significant (P<0.05) enhancement in mRNA levels of $Er\alpha$ and $Er\beta$ in the PRP auto-located groups compared to the PCOS-sole groups. More IHC analyses showed similar results, representing the elevated number of $Er\alpha$ and $Er\beta$ -positive cells per 1 mm² of tissue in the PRP auto-located groups versus

the PCOS-sole animals (Fig.3A-E).

Platelet-rich plasma decreased PCOS-induced *c-Myc* overexpression

The PCOS-sole animals exhibited increased expression of *c-Myc* compared to control group. Meanwhile, the animals of PRP auto-located groups showed diminished expression of *c-Myc* versus PCOS-sole groups. Accordingly, lower mRNA level and *c-Myc*-positive cells distribution were observed in PRP auto-located animals (Fig.4A-D).



Fig.3: IHC staining and RT-PCR results for $\text{Er}\alpha$ and $\text{Er}\beta$. **A.** See decreased $\text{Er}\alpha$ -positive reactions in the PCOS-sole group, while it is increased in the PRP-treated groups. Note the increased $\text{Er}\beta$ -positive cells in the PRP-treated group (30 days after PCOS-induction), **B.** Mean ± SD of $\text{Er}\alpha$ (a vs. b; P=0.01, a vs. c; P=0.03, b vs. c; P=0.03), **C.** $\text{Er}\beta$ -positive cells per 1 mm² of tissue in different groups (n=6) (a vs. b, c; P=0.001), **D.** Electrophoresis photomicrographs of $\text{Er}\alpha$ and $\text{Er}\beta$ mRNA in different groups, and **E.** Density of $\text{Er}\alpha$ and $\text{Er}\beta$ mRNA levels in ovarian tissue, measured by densitometry and normalized to β -Actin mRNA expression level (a vs. b; P=0.02, a vs. c; P=0.03). Arrows are representing positive reaction for $\text{Er}\alpha$ and $\text{Er}\beta$ antibodies. All data are represented in mean ± SD (n=6). Different letters represent significant differences (P<0.05) between groups (scale bar: 300 µm). IHC; Immunohistochemical, RT-PCR; Reverse transcription-polymerase chain reaction, ER; Estrogen receptor, PCOS; Polycystic ovarian syndrome, CF; Cystic follicle, CL; Corpus luteum, and PRP; Platelet-rich plasma.

PRP-treated

Day 30

Jav 15

5 0



Α



Fig.4: IHC staining and RT-PCR results for c-Myc. **A.** See increased c-Myc-positive cells in the PCOS-sole groups vs. the control group. PRP-treated sections represent reduced c-Myc-positive cells compared to the PCOS-sole groups, **B.** Mean \pm SD of c-Myc-positive cells per 1 mm² of tissue in different groups (n=6) (a vs. b; P=0.01, a vs. c; P=0.01, b vs. c; P=0.02), **C.** Electrophoresis photomicrographs of *c-Myc* mRNA in different groups, and **D.** Density of *c-Myc* mRNA levels in ovarian tissue, measured by densitometry and normalized to β -Actin mRNA expression level (a vs. b, c; P=0.01). Arrows are representing positive reaction for c-Myc antibody. All data are represented in mean \pm SD (n=6). Different letters represent significant differences (P<0.05) between groups (scale bar: 300 µm). IHC; Immunohistochemical, RT-PCR; Reverse transcription-polymerase chain reaction, PCOS; Polycystic ovarian syndrome, and PRP; Platelet-rich plasma.

Platelet-rich plasma enhanced ovarian antioxidant status

To estimate the ovarian antioxidant potential, TAC, MDA, SOD and CSG-px levels were analyzed. Observations showed significant (P<0.05) reduction in TAC, SOD and GSH-px levels of ovaries in the PCOS-sole group versus the control animals, while, ovarian MDA content was increased in the PCOS-sole groups compared to the control group. In contrast, those animals in the PRP auto-located groups exhibited remarkable (P<0.05) reduction in MDA content and significant (P<0.05) enhancement in TAC, SOD and GSH-px levels versus the PCOS-sole group. The data for antioxidant

profile are presented in Table 2.

B-Actin

310 bp>

Platelet-rich plasma ameliorated PCOS-induced hormonal imbalance

The PCOS-sole animal groups showed increased serum levels of FSH, LH, testosterone and androstenedione as well as diminished levels of estrogen and progesterone compared to control group. However, the animals of PRP auto-located groups exhibited diminished serum levels of FSH, LH, testosterone and androstenedione. Moreover, serum estrogen and progesterone levels were increased in the PRP auto-located groups in comparison to the PCOS-sole animals (P<0.05). The data for hormonal profile are presented in Table 3.

Table 2: Serum hormone levels in different groups						
Groups	Estrogen (pg/ml)	Progesterone (pg/ml)	Testosterone (ng/ml)	Androstenedione (ng/ml)	FSH (ng/ml)	LH (ng/ml)
Control	$72.25\pm19.00^{\mathrm{a}}$	66.90 ± 4.33^{a}	$0.68\pm0.34^{\rm a}$	$0.44\pm0.07^{\rm a}$	$1.24\pm0.12^{\rm a}$	0.75 ± 0.10^{a}
PCOS-15 D	$20.33\pm7.50^{\text{b}}$	$12.90\pm2.10^{\mathrm{b}}$	$2.21\pm0.62^{\rm b}$	$0.93\pm0.08^{\text{b}}$	$2.45\pm0.51^{\text{b}}$	$1.81\pm0.51^{\text{b}}$
PRP-treated 15 D	$48.64\pm5.68^{\circ}$	$60.45\pm8.91^{\text{a}}$	$0.72\pm0.22^{\rm a}$	$0.48\pm0.13^{\rm a}$	$1.28\pm0.09^{\rm a}$	$1.02\pm0.26^{\rm a}$
PCOS-30 D	$21.37\pm6.99^{\mathrm{b}}$	$10.77\pm1.45^{\mathrm{b}}$	$1.67\pm0.56^{\text{b}}$	$1.14\pm0.19^{\text{b}}$	$2.84\pm0.43^{\text{b}}$	$1.94\pm0.34^{\rm b}$
PRP-treated 30 D	56.37 ± 3.21°	$64.33\pm6.41^{\rm a}$	$0.64\pm0.16^{\rm a}$	$0.54\pm0.10^{\rm a}$	1.23 ± 0.11^{a}	$0.88 \pm 0.10^{\mathrm{a}}$

Data are presented as mean ± SD. Different letters represent significant differences (P<0.05) between data in the same row (n=6). 15 D; 15 days after PCOS-induction, 30 D; 30 days following PCOS-induction, FSH; Follicle stimulating hormone, LH; Luteinizing hormone, PCOS; Polycystic ovarian syndrome, and PRP; Platelet-rich plasma.

Groups	TAC (mMol/mg protein)	MDA (mMol/mg protein)	SOD (U/ml)	GSH-px (U/ml)
Control	1.77 ± 0.43^{a}	$0.85\pm0.10^{\rm a}$	126.66 ± 7.35^{a}	120.00 ± 21.01^{a}
PCOS-15 D	$0.45\pm0.01^{\rm b}$	$2.66\pm0.34^{\text{b}}$	$37.66 \pm 16.25^{\text{b}}$	$63.10\pm14.21^{\mathrm{b}}$
PRP-treated 15 D	$0.94\pm0.05^{\circ}$	$2.18\pm0.06^{\rm c}$	$93.33\pm7.02^{\circ}$	115.74 ± 21.37^{a}
PCOS-30 D	$0.59\pm0.02^{\rm d}$	$3.77\pm0.29^{\rm d}$	$34.00\pm9.64^{\rm b}$	$51.44\pm4.25^{\mathrm{b}}$
PRP-treated 30 D	$1.26\pm0.06^{\rm e}$	$1.12\pm0.150^{\mathrm{a,c}}$	$96.34\pm8.34^{\circ}$	$116.73 \pm 14.37^{\rm a}$

Data are presented as mean ± SD. Different letters represent significant differences (P<0.05) between data in the same row (n=6). 15 D; 15 days after PCOS-induction, 30 D; 30 days following PCOS-induction, TAC; Total antioxidant capacity, MDA; Malondialdehyde, SOD; Superoxide dismutase, GSH-px; Glutathione peroxidase, PCOS; Polycystic ovarian syndrome, and PRP; Platelet-rich plasma.

Discussion

Considering cross-links between oxidative stress, hyperandrogenemia, insulin resistance and PCOS, the present study was performed to uncover the ameliorative role of PRP against PCOS-induced/related pathogenesis in animal models. Our findings showed that, auto-locating PRP significantly improved ovarian antioxidant status, down-regulated androgen synthesis and up-regulated follicular survival as well as ovulation. Moreover serum estrogen level and expression of $Er\alpha$ and $Er\beta$, as important elements in follicular growth/atresia, were evaluated after PRP auto-location. Observations revealed that PRP significantly enhanced serum estrogen and progesterone levels and up-regulated ERs expression. Finally, considering the prooven pro-apoptotic role of *c-Myc* in theca interna of atretic follicles, as well as peripheral theca lutein cells, *c-Myc* mRNA level and *c-Myc*-positive cells distribution/1 mm² of ovarian tissue were evaluated. The PRP auto-located groups showed a remarkable reduction in *c-Myc* expression versus PCOS-sole animals.

It has been well-established that in majority of cases (especially in the models with hyperandrogenemia), PCOS associates with insulin resistance and severe oxidative stress (30, 31). To understand the subject, it should be noted that hyperglycemia and higher levels of free fatty acid following insulin resistance initiate the

oxidative stress by producing higher amounts of free radicals (32). On the other hand, positive correlation between oxidative stress and elevated androgen levels has been discovered in PCOS (33). Minding the androgen boosting effect of free radicals (34) as well as ameliorative effect of PRP on hyperandrogenemia and oxidative stress, serum androgen levels and ovarian antioxidant status were analyzed. Our findings showed that auto-locating PRP significantly diminished serum testosterone and androstenedione levels, improved ovarian TAC level and diminished lipid peroxidation ratio. On the other hand, any reduction in tissue levels of antioxidant enzymes, including SOD, GSH-px and catalase has been reported to initiate and promote oxidative stress in ovarian tissue (35). To show alterations, we assessed tissue levels of SOD and GSH-px. Observations revealed that PRP significantly enhanced PCOS-reduced GSH-px and SOD levels. Based on biochemical results, PRP could fairly up-regulate the ovarian GSH-px and SOD levels. Indeed, pathologically-produced oxidative stress results in severe damages at cellular levels of DNA, RNA, protein and lipid (36). Thus, we assessed RNA damage and MDA levels as biomarkers for oxidative stress-induced damages. Based on biochemical findings, PRP auto-location significantly diminished mRNA damage and reduced ovarian MDA content. Taking all these findings together, we can suggest that PRP induces antiandrogenic and

antioxidant effects, at least in the case of experimentallyinduced hyperandrogenic PCOS. In line with this issue and considering the boosting effect of antioxidants on meaningful follicular growth, the complementary and antioxidant chemicals are lastly used to manage/reduce the PCOS-induced pathogenesis. Consistently, various studies showed that administrating antioxidant agents are able to potentially improve insulin sensitivity and enhance the ovarian antioxidant potential in women with PCOS (37, 38).

PCOS up-regulates serum gonadotropin levels and significantly diminishes the estrogen and progesterone synthesis versus control animals and/or fertile women (4). In corroboration with those reports, the animals in PCOSsole groups showed higher serum LH and FSH levels, in addition to lower levels of estrogen and progesterone versus the control group. In contrast, PRP auto-location reversed the condition by reducing serum LH and FSH levels, and up-regulating estrogen and progesterone concentrations. In line with this, it has been illustrated that estrogen inflicts the GC proliferation, oocyte development, maintains the follicular survival (from atresia), promotes the ovarian angiogenesis (8, 9) and finally by binding to its nuclear receptor ($Er\alpha$ and $Er\beta$) stimulates various growth factors secretion, such as IGF and EGF, resulting in follicular survival (14). However, any reduction in $Er\alpha$ expression results in a failed follicular maturation and/or ovulation and hemorrhagic cysts formation. In addition, the failed $Er\beta$ expression leads to chronic anovulation (8). Thus, we can suggest that diminished estrogen secretion in PCOSsole groups impressively inflicted follicular atresia, which ultimately resulted in an impaired ovulation. Considering significant up-regulation of follicular growth as well as diminished atresia in PRP auto-located groups, we can suggest that PRP improves follicular growth by upregulating the estrogen secretion and enhancing the $Er\alpha$ and $Er\beta$ expressions. Aside these hypotheses, it should be considered that PRP, by preserving the gonadotropins secretion, might restore the ovarian-hypophysis hormonal disruption and, by up-regulating the estrogen synthesis, promoted follicular cells proliferation and oocyte development. All of these evidences thereafter promote follicular growth and accelerate successful ovulation (marked with increased corpora lutea generation and progesterone level in PRP auto-located groups). The role of growth hormones in early (FSH-independent follicular development) and late (cell proliferation and inhibiting apoptosis) folliculogenesis should not be ignored (39). As PRP potentially contains several growth factors, it would be more logic to suggest that the ameliorative effect of PRP may partially depend on several growth hormones, which could be assessable in ovaries following PRP autolocation.

Massive expression of c-Myc protein in GCs, theca interna of atretic follicles and peripheral theca lutein cells confirm the c-Myc-induced pro-apoptotic characteristic (11). Our RT-PCR and IHC analyses showed increased *c-Myc* expression in PCOS-sole groups versus control

animals. However, the animal of PRP auto-located groups exhibited a diminished expression of *c-Myc*. In order to understand the subject, contrary roles of c-Myc should be highlighted. Indeed, c-Myc, under certain conditions, exerts completely opposite features. Accordingly, the estrogen (at physiologic levels) by targeting the ERs (especially $Er\alpha$), stimulates the follicular growth through induction of G1- to S-phase transition. Actually, current induction is mainly associated with rapid and direct up-regulation of c-Myc, controlling cyclin D1 expression, cyclin-dependent kinase (CDK) activation and phosphorylation of retinoblastoma proteins (40). In contrast, c-Myc overexpression and/or inappropriate expression is sufficient to induce/promote apoptosis in GCs, theca interna of atretic follicles and peripheral theca lutein cells (10, 11). All of these evidences inflict atresia. Taking all together, we can conclude that diminished estrogen synthesis, associated with decreased expression of ERs in PCOS-sole groups, may trigger c-Myc overexpression, leading to impressive apoptosis at follicular level. However, ameliorated estrogen synthesis and up-regulated ERs expression in PRP-auto-located groups could fairly adjust the PCOS-increased c-Myc level. Diminished follicular atresia in PRP auto-located groups confirms this hypothesis.

Although ameliorated follicular growth, enhanced ovulation ratio (marked with higher corpora lutes), upregulated antioxidant status and balanced hormonal levels are illustrated in the current study, there are some limitations in this study -including sample size in terms of quantity, focusing on aromatization, angiogenesis and insulin resistance of animals- which should be considered in the future studies.

Conclusion

Our preliminary data showed that auto-locating PRP fairly ameliorates PCOS-induced pathogenesis. Accordingly, it is able to suppress androgen oversynthesis and ameliorate hormonal imbalance, in addition to improvement of ovarian antioxidant status as well as inhibiting c-Myc overexpression. It can ultimately enhance ovulation ratio. Considering these findings and minding high amounts of different growth factors in PRP, auto-location of this factor could be considered as a new method for PCOS subjects.

Acknowledgements

This study was financially supported by Ahar Islamic Azad University and the authors wish to thank, Departments of Basic Sciences and Biochemistry, Urmia and Tabriz Universities, and also AYANDEH Lab. Co. for laboratory supports. The authors declare no conflict of interest.

Authors' Contributions

M.R.; Concept and design of the study, analysis and interpretation of data, final approval of the version to be

submitted. Gh.D.; Study design, drafting the manuscript and revising it critically in terms of intellectual content. S.S.A.; Participating in laboratory experiment and data analyses. All authors read and approved the final manuscript.

References

- 1. Vrbikova J, Hainer V. Obesity and polycystic ovary syndrome. Obes Facts. 2009; 2(1): 26-35.
- Rotterdam ESHRE/ASRM-Sponsored PCOS consensus workshop group. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). Hum Reprod. 2004; 19(1): 41-47.
- Goodarzi MO, Dumesic DA, Chazenbalk G, Azziz R. Polycystic ovary syndrome: etiology, pathogenesis and diagnosis. Nat Rev Endocrinol. 2011; 7(4): 219-231.
- Banaszewska B, Spaczynski RZ, Pelesz M, Pawelczyk L. Incidence of elevated LH/FSH ratio in polycystic ovary syndrome women with normo- and hyperinsulinemia. Rocz Akad Med Bialymst. 2003; 48: 131-134.
- Dupont S, Krust A, Gansmuller A, Dierich A, Chambon P, Mark M. Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes. Development. 2000; 127(19): 4277-4291.
- Carreau S. Germ cells: a new source of estrogens in the male gonad. Mol Cell Endocrinol. 2001; 178(1-2): 65-72.
- Sar M, Welsch F. Differential expression of estrogen receptor-beta and estrogen receptor-alpha in the rat ovary. Endocrinology. 1999; 140(2): 963-971.
- Drummond AE, Fuller PJ. Ovarian actions of estrogen receptorbeta: an update. Semin Reprod Med. 2012; 30(1): 32-38.
- Lee HR, Kim TH, Choi KC. Functions and physiological roles of two types of estrogen receptors, ERα and ERβ, identified by estrogen receptor knockout mouse. Lab Anim Res. 2012; 28(2): 71-76.
- Hoffman B, Liebermann DA. Apoptotic signaling by c-MYC. Oncogene. 2008; 27(50): 6462-6472.
- Nandedkar TD, Dharma SJ. Expression of bcl(xs) and c-myc in atretic follicles of mouse ovary. Reprod Biomed Online. 2001; 3(3): 221-225.
- Evan GI, Littlewood TD. The role of c-myc in cell growth. Curr Opin Genet Dev. 1993; 3(1): 44-49.
- Murri M, Luque-Ramirez M, Insenser M, Ojeda-Ojeda M, Escobar-Morreale HF. Circulating markers of oxidative stress and polycystic ovary syndrome (PCOS): a systematic review and meta-analysis. Hum Reprod Update. 2013; 19(3): 268-288.
- Zuo T, Zhu M, Xu W. Roles of Oxidative Stress in Polycystic Ovary Syndrome and Cancers. Oxid Med Cell Longev. 2016; 2016: 8589318.
- Savic-Radojevic A, Bozic Antic I, Coric V, Bjekic-Macut J, Radic T, Zarkovic M, et al. Effect of hyperglycemia and hyperinsulinemia on glutathione peroxidase activity in non-obese women with polycystic ovary syndrome. Hormones (Athens). 2015; 14(1): 101-108.
- Bhanot S, Alex JC. Current applications of platelet gels in facial plastic surgery. Facial Plast Surg. 2002; 18(1): 27-33.
- Bose B, Balzarini MA. Bone graft gel: autologous growth factors used with autograft bone for lumbar spine fusions. Adv Ther. 2002; 19(4): 170-175.
- DelRossi AJ, Cernaianu AC, Vertrees RA, Wacker CJ, Fuller SJ, Cilley JH Jr, et al. Platelet-rich plasma reduces postoperative blood loss after cardiopulmonary bypass. J Thorac Cardiovasc Surg. 1990; 100(2): 281-286.
- Margolis DJ, Kantor J, Santanna J, Strom BL, Berlin JA. Effectiveness of platelet releasate for the treatment of diabetic neuropathic foot ulcers. Diabetes Care. 2001; 24(3): 483-488.
- Martins RP, Hartmann DD, de Moraes JP, Soares FA, Puntel GO. Platelet-rich plasma reduces the oxidative damage determined by a skeletal muscle contusion in rats. Platelets. 2016; 27(8): 784-790.

- Honnma H, Endo T, Henmi H, Nagasawa K, Baba T, Yamazaki K, et al. Altered expression of Fas/Fas ligand/caspase 8 and membrane type 1-matrix metalloproteinase in atretic follicles within dehydroepiandrosterone-induced polycystic ovaries in rats. Apoptosis. 2006; 11(9): 1525-1533.
- Messora MR, Nagata MJH, Furlaneto FAC, Dornelles RCM, Bomfim SRM, Deliberador TM, et al. A standardized research protocol for platelet-rich plasma (PRP) preparation in rats. RSBO. 2011; 8(3): 299-304.
- Darzynkiewicz Z, Bedner E, Li X, Gorczyca W, Melamed MR. Laser-scanning cytometry: a new instrumentation with many applications. Exp Cell Res. 1999; 249(1): 1-12.
- Adibnia E, Razi M, Malekinejad H. Zearalenone and 17 β estradiol induced damages in male rats reproductionpotential; evidence for ERα and ERβ receptors expression and steroidogenesis. Toxicon. 2016; 120: 133-146.
- Wiren KM, Chapman Evans A, Zhang XW. Osteoblast differentiation influences androgen and estrogen receptor-alpha and -beta expression. J Endocrinol. 2002; 175(3): 683-694.
- 26. Li XF, Wang SJ, Jiang LS, Dai LY. Gender- and region-specific variations of estrogen receptor α and β expression in the growth plate of spine and limb during development and adulthood. Histochem Cell Biol. 2012; 137(1): 79-95.
- Pant N, Srivastava SP. Testicular and spermatotoxic effects of quinalphos in rats. J Appl Toxicol. 2003; 23(4): 271-274.
- Niehaus WG Jr, Samuelsson B. Formation of malonaldehyde from phospholipid arachidonate during microsomal lipid peroxidation. Eur J Biochem. 1968; 6(1): 126-130.
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951; 193(1): 265-275.
- Salvado L, Palomer X, Barroso E, Vazquez-Carrera M. Targeting endoplasmic reticulum stress in insulin resistance. Trends Endocrinol Metab. 2015; 26(8): 438-448.
- Banaszewska B, Pawelczyk L, Spaczynski RZ, Dziura J, Duleba AJ. Effects of simvastatin and oral contraceptive agent on polycystic ovary syndrome: prospective, randomized, crossover trial. J Clin Endocrinol Metab. 2007; 92(2): 456-461.
- 32. Pisoschi AM, Pop A. The role of antioxidants in the chemistry of oxidative stress: a review. Eur J Med Chem. 2015; 97: 55-74.
- Yang Y, Qiao J, Li R, Li M-Z. Is interleukin-18 associated with polycystic ovary syndrome? Reprod Biol Endocrinol. 2011; 9(1): 7.
- González F, Sia CL, Shepard MK, Rote NS, Minium J. Hyperglycemia-induced oxidative stress is independent of excess abdominal adiposity in normal-weight women with polycystic ovary syndrome. Hum Reprod. 2012; 27(12): 3560-3568.
- 35. Fardoun RZ. The use of vitamin E in type 2 diabetes mellitus. Clin Exp Hypertens. 2007; 29(3): 135-148.
- Molavi M, Razi M, Malekinejad H, Amniattalab A, Rezaie H. Vitamin E improved cypermethrin-induced damages in the ovary of rats; evidence for angiogenesis and p53 involvement. Pestic Biochem Physiol. 2014; 110: 27-35.
- Amini L, Tehranian N, Movahedin M, Ramezani Tehrani F, Ziaee S. Antioxidants and management of polycystic ovary syndrome in Iran: a systematic review of clinical trials. Iran J Reprod Med. 2015; 13(1): 1-8.
- Carmina E, Koyama T, Chang L, Stanczyk FZ, Lobo RA. Does ethnicity influence the prevalence of adrenal hyperandrogenism and insulin resistance in polycystic ovary syndrome? Am J Obstet Gynecol. 1992; 167(6): 1807-1812.
- Giovanni Artini P, Monteleone P, Parisen Toldin MR, Matteucci C, Ruggiero M, Cela V, et al. Growth factors and folliculogenesis in polycystic ovary patients. Expert Rev Endocrinol Metab. 2007; 2(2): 215-223.
- Wang C, Mayer JA, Mazumdar A, Fertuck K, Kim H, Brown M, et al. Estrogen induces c-myc gene expression via an upstream enhancer activated by the estrogen receptor and the AP-1 transcription factor. Mol Endocrinol. 2011; 25(9): 1527-1538.

Total Antioxidant Capacity; A Potential Biomarker for Non-Invasive Sex Prediction in Culture Medium of Preimplantation Human Embryos

Nahid Nasiri, M.Sc.¹, Leila Karimian, M.Sc.¹, Fatemeh Hassani, M.Sc.¹, Hamid Gourabi, Ph.D.²,

Hiva Alipour, Ph.D.³, Zahra Zolfaghari, M.Sc.⁴, Poopak Eftekhari-Yazdi, Ph.D.^{1*}

1. Department of Embryology, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran

Department of Genetics, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran
 Biomedicine Group, Department of Health Science and Technology, Faculty of Medicine, Aalborg University, Aalborg, Denmark
 Department of Epidemiology and Reproductive Health, Reproductive Epidemiology Research Center, Royan Institute for Reproductive Biomedicine. ACECR, Tehran, Iran

*Corresponding Address: P.O.Box: 16635-148, Department of Embryology, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran Email: eftekhari@royaninstitute.org

Received: 10/July/2018, Accepted: 15/September/2018

Abstract

Objective: The presence of a sex related metabolic difference in glucose utilization and, on the other hand, different developmental kinetic rates in human preimplantation embryos, has been previously observed, however, the correlation between these two events is unknown. Oxidative stress (OS) induced by higher glucose consumption appears to be a possible cause for the delayed development rate in female embryos. We examined the correlation between glucose consumption and total antioxidant capacity (TAC) concentration in individual embryo culture media for both male and female embryos.

Materials and Methods: In this cross-sectional study, we evaluated high quality embryos from 51 patients that underwent intracytoplasmic sperm injection (ICSI) and preimplantation genetic diagnosis (PGD) at the Royan Institute between December 2014 and September 2017. The embryos were individually cultured in G-2[™] medium droplets at days 3-5 or 48 hours post PGD. We analysed the spent culture media following embryo transfer for total antioxidant capacity (TAC) and any remaining glucose concentrations through fluorometric measurement by chemiluminecence system which indirectly was used for measurement of glucose consumed by embryos.

Results: The results showed that female embryos consumed more glucose which was associated with decreased TAC concentration in their culture medium compared to male embryos. The mean of glucose concentration consumed by the female embryos ($30.7 \pm 4.7 \text{ pmol/embryo/hour}$) was significantly higher than that of the male embryos ($25.3 \pm 3.3 \text{ pmol/embryo/hour}$) (P<0.001). There were significantly lower levels of TAC in the surrounding culture medium of female embryos ($22.60 \pm 0.19 \text{ nmol/µl}$) compared with male embryos ($24.74 \pm 0.27 \text{ nmol/µl}$, P<0.01).

Conclusion: This finding highlighted the utilization of sex dependent metabolic diversity between preimplantation embryos for non-invasive sex diagnosis and suggests the TAC concentration as a potential noninvasive biomarker for prediction of sex.

Keywords: Antioxidant, Culture Medium, Glucose, Human Embryo, Sexuality

Cell Journal (Yakhteh), Vol 21, No 3, October-December (Autumn) 2019, Pages: 253-258 _

Citation: Nasiri N, Karimian L, Hassani F, Gourabi H, Alipour H, Zolfaghari Z, Eftekhari-Yazdi P. Total antioxidant capacity; a potential biomarker for noninvasive sex prediction in culture medium of preimplantation human embryos. Cell J. 2019; 21(3): 253-258. doi: 10.22074/cellj.2019.6115.

Introduction

Throughout the past few decades, preimplantation embryo physiology and its related technologies (proteomics and metabolomics) have been employed with multiple purposes. Better recognition of embryo properties, improvement of embryo culture media, and selection of the most viable embryos for transfer via in vitro fertilization/intracytoplasmic sperm injection (IVF/ICSI) cycles are the most important goals (1, 2). Assessment of embryo metabolism has been suggested recently for diagnosis of sex related differences between preimplantation embryos. These differences are attributed to different X chromosome content among males and females during the finite period-between embryonic genome activation and excess X chromosome inactivation (3). During this period, the presence of two transcriptionally active X chromosomes in females forms the basis for the different proteome and physiologies between males and females (4). These differences can lead to sex dimorphism including different concentrations of X-linked enzymes that are primarily involved in nutrient utilization and energy metabolism (5). There are numerous reports about the feasibility for assessing these metabolic differences in embryonic culture media without the need for embryo manipulation and increased time expenditure (4, 5) and in the future this rapid, non-invasive approach, may be able to replace preimplantation genetic diagnosis (PGD) which involves the biopsy of a blastomere at the cleavage/blastocyst stage followed by the identification of the sex chromosomes. PGD is considered an invasive, time consuming technique for sex identification in preimplantation human embryos prior to their transfer to the uterus (4). In order to quantify such physiological differences, we can analyze the X chromosome dependent events in embryo blastomeres. Based on possible data analysis, it may be feasible to predict an embryo's sex

without the use of PGD. Among the various metabolites, more attention has been paid to glucose which presents at high concentrations in the female reproductive tract during early embryo development. It has been suggested that glucose has a greater relationship with embryo sex compared to other metabolites (6). Previous reports discussed the different schema of glucose utilization between male and female preimplantation embryos (4). Initial studies reported increased glucose and pyruvate uptake by male embryos compared to females (7), whereas more recent studies reported increased glucose uptake by female embryos (5, 6).

The rate of glucose metabolism may change due to X-choromosome dosage mainly because the Glucose -6- phosphate dehydrogenase (G6PD), that catalyzes the principal glucose metabolism pathway (pentose phosphate pathway, PPP), is encoded by X-chromosome, and this double concentration in female blastocysts compared to male blastocysts (8). On the other hand, a slower development rate of female preimplantation embryos in the in vitro culture (IVC) has been frequently observed (9, 10). According to these studies, delays in development have shown significant correlation with increased glucose consumption (6). Further glucose consumption and hyperglycemia are commonly associated with reduced or delayed blastocyst formation (11), lower implantation rate (12), reduced live birth rate, and decreased fertility due to induction of metabolic disorders (13).

Several mechanisms proposed for such disorders attributed to high glucose consumption include increased cell apoptosis, glucose transport perturbation, and mitochondrial dysfunction (6), all of which may induce oxidative stress (OS) followed by increased reactive oxygen species (ROS) production (14). However, although female embryos experience a slower rate of development along with increased glucose consumption compared to male embryos, the correlation of the sex related glucose consumption with induced OS in culture medium that surrounds the embryo is unknown. Therefore, the present study is the first to investigate the relationship between glucose uptake on days 4 and 5 by individually cultured human embryos and the total antioxidant capacity (TAC) concentrations in their culture mediums and applying it to predict embryo sex.

Materials and Methods

Participants

This cross-sectional study included 60 cleavage-stage embryos from 51 fertile couples at the Royan Institute, Tehran, Iran, between December 2014 and September 2017. All 51 couples signed a written informed consent for the collection of residue embryo culture media after embryo transfer. For each couple prior to starting the treatment, a comprehensive counseling was provided by a reproductive endocrinologist and clinical geneticist. Thirty eight embryos from 30 women referred to Royan Institute for ICSI-PGD as an indication for the risk of sex linked diseases, and 22 embryos from 13 patients that underwent ICSI-PGD because of sex selection decision for family balancing (i.e., for patients who already had at least two children of one sex and desired a child of the other sex).

We performed ICSI in order to achieve high fertilization rates in included patients and prevent the formation of sperm bound to the zona pellucida during the blastomere biopsy. The local Ethics Committee of Royan Institute granted approval for this study (reference number: EC/91/1033). All data were collected following patient informed consent and protection of patient confidentiality. Throughout the duration of this study, all gamete and embryo culture media and handling protocols, as well as embryology lab staff remained constant.

Ovarian hyperstimulation

Patients included in this study underwent standard controlled ovarian stimulation that consisted of suppression of pituitary gonadotropin secretion by subcutaneous injection (500 mg/d) of the gonadotropin releasing hormone (GnRH) agonist, buserelin acetate (Suprefact, HoechstAG, Germany). Patients received these injections during the mid-luteal phase of the preceding ovarian cycle (day 21). We conducted this study from August 2014 to September 2015 at the Royan Institute's Assisted Conception Unit. Once ovarian suppression was confirmed, ovarian stimulation was initiated with recombinant follicle stimulating hormone (FSH, Gonal F, SC injection, 150 IU/d, Serono, Switzerland). When the average diameter of at least three follicles reached 18 mm, each patient received a single injection of human chorionic gonadotropin (hCG) (10000 IU, Pregnyl, Organon, Netherlands). Oocyte collection was performed by standard ultrasound guided follicular puncture at 36 hours after the hCG trigger.

Intracytoplasmic sperm injection and embryo culture

At 1 hour after oocyte retrieval, we selected morphologically ideal oocytes for ICSI. Oocytes were maintained in G-IVFTM medium (Vitrolife, Sweden) for approximately 2 hours before ICSI. The spermatozoa were prepared using density gradient centrifugation (AllGrad®, LifeGlobal, US). For ICSI, the oocytes were initially incubated in 80 IU/ml hyaluronidase for less than 30 seconds and cumulus cells were stripped off the oocyte by gentle pipetting. Fertilization was confirmed at 16 to 17 hours after ICSI, by the presence of two pronuclei and a second polar body. Zygotes were placed individually in 20 µl fresh G-1TM medium (Vitrolife) supplemented with 10% recombinant human serum albumin (HSAsolutionTM, Vitrolife) under oil (OVOILTM, Vitrolife) for a 48 hours culture.

Embryo biopsy and preimplantation genetic diagnosis

Embryo biopsy was performed on day 3 after fertilization. Embryos of Grade A, B or C, that had >6 cells and <20% fragmentation were biopsied. For each selected embryo

the blastomeres were checked for the presence of nuclei. Each embryo was placed in a droplet of Ca²⁺and Mg²⁺-free medium (G-PGDTM, Vitrolife) and the zona pellucida was perforated using a Nikon TE300 inverted microscope (Nikon, Japan) equipped with a zona infrared laser optical system (ZILOS, Hamilton-Thorn, Beverg, MA) with a 1.48-mm infrared diode laser beam. One blastomere was gently aspirated with an aspiration pipette (\pm 35 µm outer diameter) and individually fixed under an inverted microscope. Sex chromosomes were assessed as previously described (15). We used DNA probes for chromosomes X and Y (Vysis, Abottmol, USA) for PGD analysis of the cells. The probe for the X chromosome was labeled with spectrum aqua and for the Y chromosome the probe was labeled with spectrum green which resulted in blue and green fluorescence, respectively. After the biopsy on day 3, embryos were individually cultured in 20 µl of G-2TM medium (Vitrolife) supplemented with 10% HSA until sex determination and transfer to uterus on day 5 (120 hours after fertilization).

Measurement of glucose and total antioxidant capacity concentrations

To evaluation of sex related differences in glucose and TAC concentrations between male and females embryos, remaining 20 μ l embryo culture media (10 μ l for each variable) from all 48 hours cultured embryos (days 3 to 5 between embryo biopsy and embryo transfer) were used after transfer of embryos.

At the time of culture media evaluation, the embryo sexuality was unknown because the researcher was not informed from the sex determination results specified by PGD. Concentration analysis was based on fluorometric measurement of any remaining glucose using a chemiluminecence system (SynergyTM H4 Hybrid Multi-Mode Microplate Reader, Biotek, USA, Ex/Em=535/587 nm) and a glucose assay kit (K618-100, Biovision) which can detect 10 pmol to 10 nmol glucose per assay.

TAC concentration was evaluated via colorimetric measurement giving a broad absorbance peak around 570 nm and a TAC assay Kit (K274-100, Biovision, USA) which its detection limit is approximately 0.1 nmol per well (or $1 \mu M$) of Trolox or TAC. Since the direct evaluation of glucose consumption by embryo is possible only through invasive techniques such as radioimmunoassay, in a non-invasive approach, we measured the amount of glucose consumed by the embryos through considering the glucose concentration that remained in culture medium after the 48 h culture period as well as concentration of glucose in the medium at the start of the incubation period (control), volume of individual embryo surrounded culture medium (20 µl) and the number of hours of embryo incubation (48 hours). In this way, for control and each embryo sample, the volume of culture medium multiplied by measured glucose concentration and the difference between the two time points (i.e. at the start

of embryo incubation and 48 hours after incubation) was the number of pmols consumed by the embryo during the incubation. Then we divided this by the number of hours of incubation and final values were obtained in pmol/embryo/hours. accordingly our proposed formula for non-invasive measurement of glucose uptake by each embryo is as fallows;

$$[G]_{e} = ([G]_{48h} \times V) - ([G]_{0} \times V))/h$$

Glucose concentration consumed per embryo (pmol/ embryo/hour): [G]_

Volume of culture medium (microliters): V

Glucose concentration at incubation time zero: [G]₀

Glucose concentration at the end of incubation time (48 hours): $[G]_{48h}$

Incubation time duration (hours): h

Statistical analysis

Comparison of quantitative variables (TAC and glucose concentrations) between the male and female groups was performed by the student's t test for independent samples in normally distributed data, as assessed by the Kolmogorov-Smirnov test. P<0.01 was considered statistically significant. All data were expressed as mean \pm standard error (SE). The statistical analysis was carried out using SPSS version 16 (SPSS Inc., Chicago, IL, USA).

Results

Table 1 shows the demographic characteristics of participants. We observed significantly higher glucose consumption by female embryos during the 48 hours embryo culture compared to the male embryos. The mean of glucose concentration consumed by the female embryos $(30.7 \pm 4.7 \text{ pmol/embryo/hour})$ was significantly higher than that of the male embryos $(25.3 \pm 3.3 \text{ pmol/embryo/hour})$, P<0.001, Fig.1).

Table 1: Demographic and clinical characteristics of patients

Included women characteristic	Male embryos	Female embryos
The relevant patient number	27	24
Women age mean (Y) (range)	33.7 ± 1.1 (23-38)	34.1 ± 0.9 (24-38)
FSH levels at baseline (IU/l)	7.2 ± 1.8	7.5 ± 2.1
Mean anti-mullerian hormone levels at baseline (µg/l)	1.8 ± 1.4	1.9 ± 1.2
Total assessed embryos in each group	30	30

Data rare presented as mean ± SE or n. FSH; Follicle stimulating hormone.



Fig.1: Total antioxidant capacity (TAC) concentration in individual culture media droplets after 48 hours culture of human post compaction embryos. ******; Significant difference from female embryos (P<0.001).

Assessment of TAC concentration in the individual embryo culture medium in terms of sex and glucose consumption showed an indirect association between glucose utilization and TAC concentration. There were significantly lower levels of TAC in the surrounding culture medium of female embryos ($22.60 \pm 0.19 \text{ nmol}/\mu$ l) compared with male embryos ($24.74 \pm 0.27 \text{ nmol}/\mu$ l, P<0.01, Fig.2).



Fig.2: The median glucose concentration consumed by embryo per hour (pmol/embryo/hour). **; Significant difference from female embryos (P<0.001).

Discussion

Our results indicated significantly more glucose consumption by female embryos on days 4 and 5 of preimplantation development compared to male embryos. This increased consumption in female embryos was concomitant with significantly lower TAC concentration in their surrounding culture medium compared with male embryos.

Animal (4) and human (16) studies previously reported

the sex related pattern of glucose consumption in preimplantation embryos. On the other hand, other studies reported varying developmental kinetic rates between male and female embryos. Female embryos experienced slower development rates, which was probably due to increased glucose uptake (17).

It seems that differential expression of X-chromosome linked genes involved in glucose metabolism, such as SOX, MnSOD, BAX and most importantly G6PD which suggest to be stress inducing factor and is significantly higher in female embryos than males, is the cause for the observed difference in developmental kinetic rate between male and female (18). Researchers proposed that OS induction followed by generation of ROS was the probable mediator between slow development rate and high glucose metabolism (19). Impaired glucose metabolism as seen in diabetes could lead to decreased superoxide dismutase (SOD) and glutathione S transferase (GST) expression as important antioxidant enzymes (20). Induced OS might alter the cell signaling pattern and metabolism (21). OS could affect the genome and epigenome in the form of DNA, RNA, proteins and microRNAs. The slow developmental kinetic rate of female embryos in the presence of OS was not the result of mitosis reduction. Rather, the impaired proportion of blastocyst inner cell mass (ICM) reduction in favor of trophectoderm (TE) enhancement could be a possible cause (22).

ROS generation, which is one main feature of aerobic metabolism and mitochondrial oxidative phosphorylation, originates from various sources both inside the embryo as well as the embryo's surrounding medium. In the embryo, reductions in mitochondrial oxygen generate ROS via multiple enzymatic mechanisms during normal metabolism; this increasing concentration of ROS can activate the antioxidant defense mechanism (21).

Cleaving embryos before compaction utilize lactate and pyrovate during glycolysis as an anaerobic metabolism, therefore the production of ROS could be minimize, whereas glucose consumption around the time of compaction employed the oxidative phosphorylation which could lead to increased production of ROS due to aerobic metabolism of glucose (5). Under such circumstances the antioxidant defense system would protect cells from damage until the over production of ROS overcome the antioxidant defense.

The counteractive antioxidant system is linked both to extra and intra embryonic circumstances. Extra embryonic conditions present as non-enzymatic antioxidants in follicular and tubal fluids, as well as the embryo culture medium. Intra embryonic protection is mainly comprised of enzymatic antioxidants (22). In the IVC systems as with *in vivo* media, the redox potential of antioxidant compounds that have ROS trapping ability is very important. A major antioxidant compound of embryo culture media is EDTA, a metal chelator, which is supposed to inhibit both enzymatic and non-enzymatic oxidation. Another known antioxidant compound in culture media, albumin, contains proxyabsorbing potency that can trap ROS. However, culture media are closed systems unlike dynamic systems such as tubal and follicular fluids present in the female genital tract which can exchange antioxidant compounds with cells (23). In this environment, progressive production of ROS results from increasing oxidative phosphorylation; glucose metabolism may induce OS which can lead to subsequent damage. Antioxidants inhibit oxidation of macromolecules via ROS removal; in this way they are subjected to oxidation (24) and concentration decline.

Our data showed significantly less TAC concentration in the culture media at 48 h post-compaction of the female embryo culture along with increased glucose utilization compared to male embryos. We could not measure the ROS content of the culture medium droplet because of the inadequate sample size (20 μ l) which was not sufficient for simultaneous determination of glucose, TAC and ROS. However, previous reports of increased ROS production attributed to further glucose metabolism indicated that the higher amount of ROS seen in the female embryo culture medium was not out of context. Therefore, we analyzed the antioxidant status of the remaining medium that surrounded the embryo in order to assess OS, for the first time, with regards to glucose uptake and embryo sexuality.

In this study, for the first time the glucose consumption by individual embryos was evaluated indirectly and noninvasively through measuring the remnant glucose in culture medium after embryo incubation period. In this way, the embryos remain intact; therefore this technique can be used for similar purposes in the clinic. However, due to the limited, closed condition of culture systems, OS induction is unavoidable. On the other hand, addition of antioxidant compounds must be logical and based on accurate observations because of the toxicity of excess chemical compounds and antioxidants. However, we have evaluated a relatively small number of embryos because of the limitations in patient inclusion criteria and the use of only one ART center for sample collection.

Conclusion

The results of this study could be of benefit in two areas-first, these results might improve knowledge of sex related metabolic differences and modification of embryo culture mediums based on embryo requirements. Second, such information following other related observations could be used for non-invasive recognition of embryo gender before transfer in IVF/ICSI cycles.

In this study, we did not predict the embryo sexuality before PGD, but we presented the potential variable that its value was significantly associated with embryo sex determined previously by PGD. We suggest that considering such metabolic variables can help us in noninvasive prediction of pre-implantation human embryo sex. However to confirm our findings as well as other observations from similar studies, it would be necessary to design detailed studies with higher numbers of samples.

Acknowledgments

This work was financially supported by Royan Institute for Reproductive Medicine, Stem Cell Biology and Biotechnology. The authors would also like to show their gratitude to the IVF and genetic labs of Royan Institute specially Mr. Arash Behbahanian and Mr. Mostafa Fakhri who provided expertise that greatly assisted the research. The authors report no conflicts of interest.

Authors' Contributions

N.N., P.E.-Y.; Participated in study design, manuscript writing and contributed to all experimental work. F.H., L.K.; Performed individual embryo culture media and data collection. Z.Z.; Analyzed the data and contributed in interpretation of data. H.G., H.A.; Contributed in interpretation of data and conclusion. All authors read and approved the final manuscript.

References

- Dominguez F, Gadea B, Mercader A, Esteban FJ, Pellicer A, Simón C. Embryologic outcome and secretome profile of implanted blastocysts obtained after coculture in human endometrial epithelial cells versus the sequential system. Fertil Steril. 2010; 93(3): 774-782. e1.
- Wei Y, Zhang H, Wang Q, Zhang C. Single cell genetics and epigenetics in early embryo: from oocyte to blastocyst. Adv Exp Med Biol. 2018; 1068: 103-117.
- Ortega NM, Winblad N, Plaza Reyes A, Lanner F. Functional genetics of early human development. Curr Opin Genet Dev. 2018; 52: 1-6.
- Gardner DK, Larman MG, Thouas GA. Sex-related physiology of the preimplantation embryo. Mol Hum Reprod. 2010; 16(8): 539-547.
- Gardner DK, Wale PL, Collins R, Lane M. Glucose consumption of single post-compaction human embryos is predictive of embryo sex and live birth outcome. Hum Reprod. 2011; 26(8); 1981-1986.
- Bermejo-Alvarez P, Roberts RM, Rosenfeld CS. Effect of glucose concentration during in vitro culture of mouse embryos on development to blastocyst, success of embryo transfer, and litter sex ratio. Mol Reprod Dev. 2012; 79(5): 329-336.
- Ray PF, Conaghan J, Winston RM, Handyside AH. Increased number of cells and metabolic activity in male human preimplantation embryos following in vitro fertilization. J Reprod Fertil. 1995; 104(1): 165-171.
- Peippo J, Farazmand A, Kurkilahti M, Markkula M, Basrur PK, King WA. Sex-chromosome linked gene expression in in-vitro produced bovine embryos. Mol Hum Reprod. 2002; 8(10): 923-929.
- Grant VJ, Chamley LW. Can mammalian mothers influence the sex of their offspring peri-conceptually? Reproduction. 2010; 140(3): 425-433.
- Ramin N, Thieme R, Fischer S, Schindler M, Schmidt T, Fischer B, et al. Maternal diabetes impairs gastrulation and insulin and IGF-I receptor expression in rabbit blastocysts. Endocrinology. 2010; 151(9): 4158-4167.
- Pantaleon M, Tan HY, Kafer GR, Kaye PL. Toxic effects of hyperglycemia are mediated by the hexosamine signaling pathway and o-linked glycosylation in early mouse embryos. Biol Reprod. 2010; 82(4): 751-758.
- Wyman A, Pinto AB, Sheridan R, Moley KH. One-cell zygote transfer from diabetic to nondiabetic mouse results in congenital malformations and growth retardation in offspring. Endocrinology. 2008; 149(2): 466-469.
- Doblado M, Moley KH. Glucose metabolism in pregnancy and embryogenesis. Curr Opin Endocrinol Diabetes Obes. 2007; 14(6): 488-493.
- 14. Bermejo-Alvarez P, Lonergan P, Rizos D, Gutiérrez-Adan A. Low

oxygen tension during IVM improves bovine oocyte competence and enhances anaerobic glycolysis. Reprod Biomed Online. 2010; 20(3): 341-349.

- Bazrgar M, Gourabi H, Eftekhari-Yazdi P, Vazirinasab H, Fakhri M, Hassani F, et al. The Effect of prolonged culture of chromosomally abnormal human embryos on the rate of diploid cells. Int J Fertil Steril. 2016; 9(4): 563-573.
- Picton HM, Elder K, Houghton FD, Hawkhead JA, Rutherford AJ, Hogg JE, et al. Association between amino acid turnover and chromosome aneuploidy during human preimplantation embryo development in vitro. Mol. Hum Reprod. 2010; 16(8): 557-569.
- Garcia-Herreros M, Aparicio IM, Rath D, Fair T, Lonergan P. Differential glycolytic and glycogenogenic transduction pathways in male and female bovine embryos produced in vitro. Reprod Fertil Dev. 2012; 24(2): 344-352.
- Spolarics Z, Peña G, Qin Y, Donnelly RJ, Livingston DH. Inherent X-linked genetic variability and cellular mosaicism unique to females contribute to sex-related differences in the innate immune response. Front Immunol. 2017; 8: 1455.
- 19. Binder NK, Hannan NJ, Gardner DK. Paternal diet-induced obesity

retards early mouse embryo development, mitochondrial activity and pregnancy health. PLoS One. 2012; 7(12): e52304.

- Verma Š, Sagar N, Vats P, Shukla KN, Abbas M, Banerjee M. Antioxidant enzyme levels as markers for type 2 diabetes mellitus. Int J Bioassays. 2013; 2 (4): 685-690.
- Forrester SJ, Kikuchi DS, Hernandes MS, Xu Q, Griendling KK. Reactive oxygen species in metabolic and inflammatory signaling. Circ Res. 2018; 122(6): 877-902.
- Lane M, McPherson NO, Fullston T, Spillane M, Sandeman L, Kang WX, et al. Oxidative stress in mouse sperm impairs embryo development, fetal growth and alters adiposity and glucose regulation in female offspring. PLoS One. 2014; 9(7): e100832.
- Market Velker BA, Denomme MM, Mann MR. Loss of genomic imprinting in mouse embryos with fast rates of preimplantation development in culture. Biol Reprod. 2012; 86(5): 143, 1-16.
- Bagchi D, Chaudhuri S, Sardar S, Choudhury S, Polley N, Lemmens P, et al. Modulation of stability and functionality of a phytoantioxidant by weakly interacting metal ions: curcumin in aqueous solution. RSC Advances. 2015; 5(124): 102516-102524.
Assessment of Short-Term Engraftment Potential of Ex Vivo Expanded Hematopoietic Stem Cells Using Normal Fetal Mouse in Utero Transplantation Model

Morteza Zarrabi, Ph.D.^{1, 2#}, Elaheh Afzal, M.Sc.^{2#}, Mohammad Hassan Asghari, D.V.M.^{3#}, Marzieh Ebrahimi, Ph.D.^{1*}

1. Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

2. Royan Stem Cell Technology, Company, Cord Blood Bank, Tehran, Iran 3. Animal Core Facility, Reproductive Biomedicine Research Center, Royan Institute for Animal Biotechnology, ACECR, Tehran, Iran

#The first three authors equally contributed to this work.

*Corresponding Address: P.O.Box: 16635-148, Department of Stem Cells and Developmental Biology, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran Email: mebrahimi@royaninstitute.org

Received: 29/April/2018, Accepted: 2/Octber/2018

Abstract

Objective: Ex vivo expansion is a promising strategy to overcome the low number of human umbilical cord blood hematopoietic stem cells (hUCB-HSCs). Although based on the obtained results in unnatural physiological condition of irradiated genetically immune-deficient mouse models, there has always been concern that the expanded cells have less engraftment potential. The purpose of this study was to investigate effect of common ex vivo expansion method on engraftment potential of hUCB-mononuclear cells (MNCs), using normal fetal mouse, as a model with more similarity to human physiological conditions.

Materials and Methods: In this experimental study, briefly, isolated hUCB-MNCs were cultured in common expansion medium containing stem cell factor, Flt3 ligand and thrombopoietin. The unexpanded and expanded cells were transplanted to the fetal mice on gestational days of 11.5-13.5. After administration of human hematopoiesis growth factors (hHGFs), presence of human CD45⁺ cells, in the peripheral blood of recipients, was assessed at various time points after transplantation.

Results: The expanded MNCs showed 32-fold increase in the expression of CD34+38⁻ phenotype and about 3-fold higher clonogenic potential as compared to the uncultured cells. Four weeks after transplantation, 73% (19/26) of expanded-cell recipients and 35% (7/20) of unexpanded-cell recipients were found to be successfully engrafted with human CD45⁺ cells. The engraftment level of expanded MNCs was significantly (1.8-fold) higher than unexpanded cells. After hHGFs administration, the level was increased to 3.2, 3.8 and 2.6-fold at respectively 8, 12, and 16 weeks of post transplantation. The increased expression of CXCR4 protein in expanded MNCs is a likely explanation for the present findings.

Conclusion: The presented data showed that expanded MNCs compared to unexpended cells are capable of more rapid and higher short-term engraftment in normal fetal mouse. It could also be suggested that in utero transplantation (IUT) of normal fetal mice could be an appropriate substitute for NOD/SCID mice in xenotransplantation studies.

Keywords: Chimerism, Cord Blood Stem Cell Transplantation, Hematopoietic Stem Cells

Cell Journal (Yakhteh), Vol 21, No 3, October-December (Autumn) 2019, Pages: 259-267 .

Citation: Zarrabi M, Afzal E, Asghari MH, Ebrahimi M. Assessment of short-term engraftment potential of ex vivo expanded hematopoietic stem cells using normal fetal mouse in utero transplantation model. Cell J. 2019; 21(3): 259-267. doi: 10.22074/cellj.2019.6006.

Introduction

Hematopoietic stem cells (HSCs) are multi-potent progenitor cells having the ability of both multi-lineage differentiation and long-term self-renewal (1). A single HSC can repopulate the entire hematopoietic system. Therefore, over the last six decades, HSC transplantation has been widely used to treat various hematological disorders and malignancies (2, 3). Although human umbilical cord blood (hUCB) is an invaluable source of HSCs for cell therapy, the limited cell dosage of HSCs in a cord blood (CB) unit results in delayed post-transplant hematologic recovery. So, CB transplantation has mostly been limited to pediatric or adults with low body weight (4). Although, ex vivo expansion is the most promising strategy for overcoming the limited number of hUCB-HSCs, there is always a concern that expanded HSCs have less potency for hematopoiesis and engraftment compared to unexpanded ones (5). Therefore, the efficacy and safety

of expanded cells must clearly be evaluated before use in clinic.

So far, significant advances in our understanding about the in vivo functions of human HSCs have resulted from development of severely immune-deficient models such as non-obese diabetic/severe combined immunodeficiency (NOD/SCID), NOD/SCID/gamma (NSG) and nude mice (6, 7). Most of these immune-deficient mice have short lifespan with difficult breeding performance and their husbandry requires specific pathogen-free (SPF) environment (8). Moreover, due to the lack of appropriate human immune cells traffic as well as lack of completely normal physiological environment, the derived data from such mice cannot be considered as a precise reflection from the real situation in our body. On the other hand, in these models, the engraftment level of human adult stem cells in the most organs other than hematopoietic system

is very low (9). Because of the drawbacks associated with abnormal physiological condition of the models, there is considerable skepticism about the obtained results from such mice. So, there is still a need to find an animal model with more similarity to the *in vivo* environment of human body which can also be accessible for all laboratories, especially for whom with limited animal facilities.

In the last two decades, for allo- or xeno-transplantation studies, in utero transplantation (IUT) model of various animal fetuses such as mice (10), dogs (11), pigs (12), monkeys (13) and sheep (14) have alternatively been used to the genetically immune-deficient mice. For example, using a sheep IUT assay, it has been shown that a non-integrating and non-replicating Sendai virus vector expressing HoxB4 gene can efficiently enhance the ex vivo expansion of hUCB-CD34⁺ cells (15). Furthermore, it has been demonstrated that treatment of pregnant sheep by busulfan (a myeloablative agent), 6 days before IUT, could improve engraftment level of human cells (16). In IUT model, before immunologic maturity of the fetus (when chimerism and donor-specific immune tolerance can be created), allo- or xeno-geneic cells intrauterine transplantation is performed. Since there is no need to use myeloablative drugs or irradiation, immature preimmune fetuses of animals could be an ideal, inexpensive and powerful models for biomedical research. In addition to its use for study basic questions in developmental and stem cell biological approaches, IUT of foreign progenitor or stem cells to the unborn fetus has potential to treat and ideally cure a number of congenital hematologic and nonhematologic disorders, prior to birth (17-19).

Although large animals allow long-term and higher level of donor cell engraftment, they do require more cumbersome facilities for maintenance and examination. Therefore, it seems that small rodents such as mouse are more useful IUT models, by supplying a larger number of animals and limited facilities. In our knowledge, a few studies used fetal mouse to investigate the *in vivo* behavior of hUCB-HSCs. In this study, we used the fetal mouse IUT model to assess the effect of common *ex vivo* expansion method on the engraftment potential of hUCB-MNCs.

Materials and Methods

Preparation of human donor cells

In this experimental study, cells were obtained from UCB samples of mothers who consented according to guidelines established by the institutional human research Ethics review Committee of Royan Institute and Royan Stem Cell Technology Company (www.rsct.ir), Iran. Animal experiment was approved by the Institutional Animal Care and Committee of Royan Institute (IR. ACECR.ROYAN.REC.1394.175).

At first, 6% hydroxyethyl starch was used to sediment CB erythrocytes. Low-density MNCs were separated by lymphoprepTM (Stemcell Technology Inc., Canada) density-gradient centrifugation at 22°C, 800 g for 30

minutes. MNCs (10⁶/well) were cultured for 10 days in the StemSpan[™] medium (Stemcell Technology Inc., Canada) containing the following human recombinant cytokines all obtained from R&D Systems (USA): stem cell factor (SCF) 100 ng/ml, Fms-like tyrosine kinase 3 ligand (Flt3L) 100 ng/ml and thrombopoietin (TPO) 50 ng/ml. Freshly isolated and expanded UCB-MNCs were stained with the following antibodies against human cells: CD34-FITC, CD38-PerCP and CXCR4-PE. Appropriate isotype controls were also used to delete non-specific background signals. All of the antibodies were purchased from BD Pharmingen[™] (USA) except CXCR4 which was obtained from BioLegend, USA. After staining, the cells were analyzed on Partec flow-cytometer (Germany) and the data were analyzed using FlowMax software. Before transplantation, the cells were labeled by PKH26 cell tracking dye (Sigma, USA) according to manufacturer's instruction and they were suspended in modified Dulbecco media containing 10% fetal bovine serum (FBS), for future use.

Colony-forming cell assay

Briefly, 2000 MNCs were suspended in 0.3 ml IMDM+2% FBS and added to a 3 ml MethoCult[™] (Stem Cell Technologies, Canada) tube for a duplicate assay. After 12-14 days of culture, each plate was scored for granulocyte macrophage colony-forming unit (CFU-GM), burst forming unit-erythroid (BFU-E), as well as granulocyte, erythroid, macrophage and megakaryocyte colony-forming unit (CFU-GEMM).

Transwell migration assay

The migration assay was performed using 24-well transwell plates (Corning Costar, USA) with 5 μ m pore filters. The upper chambers were loaded with freshly isolated or 10 days expanded MNCs (10⁶ cells) in 100 μ l medium, while StemSpan medium and 100 ng/ml stromal cell derived factor-1 (SDF-1, R&D Systems) were placed into the lower chamber. After 4 hours incubation at 37°C, the migrated cells to the lower side of the filter were collected and counted.

In utero stem cell transplantation

NMRI pregnant mice were supplied by center of experimental animals of Royan Institute (Iran). Briefly, on embryonic days E11.5- E13.5, the pregnant mice were anesthetized by isoflurane inhalation and the uterine horns were exteriorized. Using handmade glass micropipettes with 70 μ m diameters, each embryo was intraperitoneally injected with 50 μ l phosphate buffer saline (PBS) containing 2-3×10⁶ hUCB-MNCs or their entire progeny following 10 days expansion. Sham group received only 50 μ l PBS. The uterine horns were returned to the abdominal cavity and the incision was closed with absorbable suture (Fig.1). The mothers were left undisturbed without bedding changes until the pups were 3 weeks old.



Fig.1: In utero transplantation of hUCB-MNCs using handmade glass micropipettes. **A.** Hair removal and sterilization of the surgical site, **B.** The uterine horns were exteriorized, **C.** 2-3×10⁶ non-cultured hUCB-MNC or their entire progeny, following 10 days expansion, were injected intraperitoneally to each recipient, and **D.** The uterine horns were returned to the abdominal cavity and the incision was closed. hUCB-MNCs; Human umbilical cord blood-mononuclear cells.

Growth factor treatment

Evaluation of chimerism was performed monthly up until age of 4 months. Considering the average weight of a mouse at the age of 4, 8, 12 and 16 weeks of age (which is respectively around 10-12, 26-35, 32-48, and 35-50 g), the recipients were treated with subcutaneous injections of human recombinant proteins all of which were obtained from R&D system: SCF (4 ng/g), Interleukin-3 (IL-3, 4 ng/g) and granulocyte-colony stimulating factor (G-CSF, 50 ng/g) for 3 times a week beginning at 3 weeks of age.

Immunostaining analyses of donor mononuclear cell

Following birth, several mice, transplanted with PKH26 labeled-MNCs, were sacrificed and frozen sections were prepared on albumin-coated slides from formaldehydefixed, optimal cutting temperature compound (OCT)embedded liver and spleen of newborn mice. The prepared slides were subjected to detect PKH26-labeled human cells using a fluorescence Nikon microscope.

Moreover, bone marrow cells were aspirated from the tibia/femur and fixed on positively charged slides with ice-cold acetone. The cells were then incubated with Anti-Human Nuclear Antigen antibody (HNA, AbCam, UK) in a humidified chamber overnight at 4°C, processing with secondary antibodies for one hour at room temperature in dark. The HNA immunostaining were observed using a fluorescence Nikon microscope.

To assess chimerism, 4 weeks after birth, 2-10 μ l of peripheral blood was collected in heparinized tubes via the tail tip excision and partial amputation of the tail. The red blood cells were lysed with ammonium chloride lysis buffer and washed with PBS. The cells were then blocked with 1% bovine serum albumin (BSA) and stained with anti-Human

CD45/34 or anti-Human Isotype Control (both from BD, USA) for 30 minutes at 4°C. After staining, at least 10^5 cells were analyzed on Partec flow-cytometer and the data were analyzed using FlowMax software. Engraftment was defined as detection of 0.2% or more human CD45 cells.

Statistical analysis

All data are expressed as the mean \pm SD. Significance of the differences between groups was determined using two-tailed Student's t test assuming unequal variances. The level of significance was set at P<0.05. The statistical analysis was carried out using SPSS version 16 (SPSS Inc., Chicago, II, USA).

Results

Increased *in vitro* proliferation and differentiation potential of the expanded human umbilical cord blood-mononuclear cells

In the first step, we sought the *in vitro* self-renewal and differentiation potential of hUCB-MNCs either before or after culture. For this purpose, freshly isolated hUCB-MNCs were cultured under very common expansion system, in serum free media containing SCF, TPO and Flt3L (STF) for 10 days. We firstly observed that number of total nucleated cells was significantly increased up to 4.3-fold after culture with STF (Fig.2). Moreover, we found that there was respectively 32 and 52.3 fold increases in the number of CD34⁺ cells and more primitive HSCs (CD34⁺CD38⁻cells).

The colony-forming rate and differentiation potential were examined by CFU-assay. As seen in Figure 2E, following the expansion, significant increases were observed in granulocytemonocyte (GM) and total CFU numbers, suggesting that the number of hematopoietic progenitor cells (HPCs) is enhanced after expansion.



Fig.2: Characterization of human donor cells. **A.** Representative dot plots of hUCB-MNCs before and after expansion, **B.** Number of the total nuclear cells was significantly increased after expansion for 10 days in STF medium (n=9, **; P<0.01), **C.** Percentage of CD34⁺ and CD34⁺ CD38⁺ cells in hUCB-MNCs at day 0 and after 10 days of expansion in STF medium, **D.** Number of CD34⁺ and CD34⁺ CD38⁺ cells were significantly increased after 10 days expansion in STF medium (n=9, *; P<0.05), and **E.** CFU number in 2000 cells of day 0 uncultured hUCB-MNCs and the progeny of an equivalent number of expanded hUCB-MNCs (n=3, ** P<0.01, ***; P<0.001). Fold expansion was calculated by dividing the absolute output number of the expanded cells expressing a specific phenotype after 10 days of culture by the respective number on day 0. hUCB-MNCs; Human umbilical cord blood-mononuclear cells, CFU; Colony forming unit, STF; SCF+TPO+FLT3L, BFU; Burst forming unit, GM; Granulocyte-macrophage, and GEMM; Granulocyte erythrocyte macrophage monocyte.

CXCR4 overexpression and increased *in vitro* homing potential of *ex vivo* expanded human umbilical cord blood-mononuclear cells

Homing and engraftment of HSCs is strictly depending on SDF-1/CXCR4 axis which can adversely be affected during *in vitro* culture (20). So, to determine effect of cytokine treatment on the homing ability of the expanded cells, expression of CXCR4 protein was evaluated before and after culture, using flow-cytometer. As shown in Figure 3A, the expanded cells expressed higher (2.3 fold) level of CXCR4 protein compared to the unexpanded cells. Moreover, regarding *in vitro* migration assay, 2.8-fold more STF-expanded cells were migrated toward the SDF-1 medium compared to the uncultured cells (Fig.3B). Therefore, it seems that hUCB-MNCs culture can increase *in vitro* homing ability of the expanded cells, which might be resulted from overexpression of CXCR4 receptor.



Outcome of in utero surgery

Figure 4A shows the surgical outcomes of 4 independent experiments. At 11.5-13.5 days of gestation, 21 pregnant mice were under surgery, out of which 5 mothers died due to surgical complications such as bleeding and prolonged anesthesia.

Totally, from mothers surviving surgery, 99 transplanted fetuses were under surgery, among which STF expanded cells, unexpanded cells and PBS were injected to respectively 38, 32 and 29 fetuses. In overall, the live birth rate was 63.6%. As seen in Figure 4B, recipients show similar viability injected by either STF expanded MNCs (68.4%) or unexpanded MNCs (62.5%). Furthermore, all of the live-born fetuses were normal and had no sign of malformations.



В

Injected material	Number of treated pregnant mice	Number of pregnancies to term	Number of injected fetuses	Number of live- born mice
Expanded MNCs	9	8	38	26
Unexpanded MNCs	9	7	32	20
Sham	9	7	29	17
Total	27	22	99	63



of the expanded cells. **A.** Representative flow-cytometer analysis of CXCR4 expression in different cells. Filled curves indicate isotype control and unfilled curves indicate labeled cells and **B.** Percentage of the STF-expanded hUBC-MNCs moved through the transwell in response to SDF-1 versus uncultured cells (day 0) (**; P<0.01, n=5). STF; SCF+TPO+FLT3L, SDF-1; Stromal cell derived factor-1, and hUCB-MNCs; Human umbilical cord blood-mononuclear cells.

Fig.4: Outcome of in utero surgery. **A.** Number of the pregnant mice and their embryos used through the whole experiment and **B.** Comparison of survival rates within expanded cell-recipients, unexpanded cell-recipients and sham. MNC; Mononuclear cells.

Short-term *in vivo* homing of human umbilical cord blood-mononuclear cells in liver and spleen of recipients

At first, transplanted fetuses were analyzed for presence of the injected cells. For this purpose, freshly isolated MNCs were labeled with a viable fluorescent membrane dye PKH26 to track after infusion (Fig.5A). Around 15-16 days of gestation, liver and spleen organs are the hematopoietic sources in the fetal mouse (21). Therefore, one set of fetuses were sacrificed 48 hours after transplantation and PKH-positive cells were tracked in the frozen sections prepared from the mentioned tissues. As shown in Figure 5B, the highest level of homing was found in spleen, while some PKH-positive cells were also detected in liver.

Expanded human umbilical cord blood-mononuclear cells have higher engraftment potential than unexpanded cells

To evaluate the engraftment, flow-cytometry analysis of the human CD45 marker was performed for the first time in peripheral blood of 3 weeks old mice (4 weeks after transplantation). To reliably eliminate background signal, the isotype control antibody was recruited. Peripheral blood cells from a normal mouse were also analyzed as an additional control for this analysis method (Fig.6A). Newborn mice were considered to be chimeric, if $\geq 0.2\%$ CD45⁺ human cells were present in their peripheral blood sample.

Totally, CD45⁺ human cells were detected in 73% (19/26) of live born mice that injected with the expanded MNCs, while only 35% (7/20) of unexpanded-cell recipients had become chimera. Previous studies have shown an increased engraftment level of human HSCs in sheep and mice, following treatment with human hematopoietic growth factors (hHGFs). Therefore, newborn recipients were treated with subcutaneous injections of different HGFs (IL3, SCF and G-CSF), 3 times a week beginning at 4 weeks after transplantation. As demonstrated in Figure 6B, at the beginning, expanded-cell recipients displayed a higher level (1.8-fold) of human engraftment compared to the unexpanded-cell recipients, while it was dramatically amplified after treatment by hHGFs. Precisely, compared to the other group, expanded-cell recipients showed 3.2-, 3.8- and 2.6-fold increases in engraftment at 8, 12 and 16 weeks after transplantation, respectively. As shown in Figure 6C, human originality and functionality of the transplanted cells were additionally confirmed by the presence of anti-HNA in the bone marrow cells of 4 months old mice treated with hHGFs. Interestingly, there was a downward trend in the engraftment of recipients that did not treat with hHGFs and injected by either expanded or unexpanded cells. So that, after 4 months, no human cell was seen in their blood (data not shown).



В

С

Α



Spleen

PKH26

Liver

Fig.5: Short-term *in vivo* homing of hUCB-MNCs. **A.** Analysis of PKH26 fluorescence of 20000 MNCs by flow-cytometer, before (unfilled curve) and after staining with PKH26 (gray filled curve) and **B.** Identification of PKH-stained hUCB-MNCs, 48 hours after infusion. Prepared frozen sections from the spleen and liver of transplanted fetuses were screened for the presence of PKH-bright cells (red: PKH26, blue: DAPI). hUCB-MNCs; Human umbilical cord blood-mononuclear cells.



Fig.6: The *ex vivo* expanded hUCB-MNCs have higher engraftment potential than unexpanded cells. **A.** Representative flow-cytometer analysis for human cell engraftment in peripheral blood of the expanded- and unexpanded-cell recipients. Peripheral blood of normal mouse was employed as negative control, **B.** Mean human engraftment levels in peripheral blood of NMRI mice fetal transplanted with expanded or unexpanded hUCB-MNCs. Mice with ≥0.2% human cells were considered chimeric, **C.** Representative flow-cytometer analysis for human cell engraftment in peripheral blood of the expanded- and unexpanded-cell recipients, and **D.** Identification of human CD45⁺ cells in the bone marrow of recipient mice. Bone marrow smears of 4-months-old transplanted mice were screened for the expression of human nuclear antigen (arrows; As mentioned they are human CD45⁺ cells). hUCB-MNCs; Human umbilical cord blood-mononuclear cells.

Discussion

In this study, overall frequency of the human donor cell engraftment in NMRI recipient mice as early as 4 weeks post transplantation was <3%. 7/20 (35%) recipients of unexpanded MNCs and 19/26 (73%) recipients of expanded MNCs were chimeric. This result indicates that hUCB-MNCs expansion produces a higher level of engraftment than freshly isolated cells. Furthermore, the average level of human cells in unexpanded-cell recipients was 0.3%, while it reached to 0.55% in expanded-cell recipients. Here, the level of human cells engrafted into NMRI mice is substantially higher than the previous reports in nondefective rodents (22-25). On the other hand, IUT of human fetal liver-MNCs or fetal BM-CD34⁺ cells into NOD/SCID mice resulted in 15% expression of human cells in 10-12% of 8 weeks old mice (26). Similar to our finding, it was previously reported that ex vivo expanded UCB-HSCs have higher engraftment ability in IUT model of sheep: 8.1% for expanded cells versus 0.1% for unexpanded cells (27). The engraftment of human CB-derived stem cells has also been evaluated in ovine fetuses (28). In this study, only 18% of lambs, IUT hUCB-CD34⁺ cells showed human cell expression up to 0.8%. In other larger species like canine model, it has been reported that IUT of 108 haploidentical CD34+ cells/kg of fetuses resulted in <1% microchimerism (29). Different rate of the chimerism as well as the level of engraftment could be related to the route of transplantation (30), quantity and quality of injected cells (31), different isolation techniques (32), different source of HSCs (33), gestational day of injection (34) and the animal species (35). The used cytokines can also affect the chimera formation (36).

Here, we used unpurified MNCs as a cell source, since unpurified MNCs contain more primitive progenitors as well as mature cells that compete for homing space with purified CD34⁺ cells (31). On top of that, we performed IUT on E11.5-E13.5, when the highest degree of chimerism was reported (37). Although treatment by hHGFs led to higher level of engraftment in both groups, the STF-expanded MNCs were always maintained higher through weeks post transplantation. The higher engraftment potential of expanded MNCs might be due to overexpression of the homing gene, *CXCR4*, following expansion. Moreover, *ex vivo* expansion provides us with higher number of progenitor and mature cells including neutrophils which can engraft more rapidly, in comparison with unexpanded CD34⁺ cells (38).

In our experiments, in the absence of treatment with hHGFs, regardless of the fact those samples were subjected or not to *ex vivo* expansion, the human cells continued to decrease until they were undetectable in the host body. This indicates that expanded cells lack the ability to long-term engraftment. This data also highlights the importance of compatibility between the hematopoietic environment of donor cells and the host body.

Conclusion

Here, we successfully demonstrated application of mouse IUT model to assess engraftment potential of hUCB-MNCs. Although the IUT model allows transplantation of xenogeneic cells without host conditioning, the frequency and levels of donor cells are significantly low. These data support the idea that despite the immaturity of fetus' immune system, there are some barriers preventing the engraftment of human cells. It seems that mainly overcoming the conflicts of hematopoietic environment as well as attenuating the immune response against the donor cells will make IUT model as an acceptable model for basic and pre-clinical research.

Acknowledgments

This study has been funded by Royan Institute (Code: 91000597) and granted partly by Royan Stem Cell Technology Company, Iran. The authors would like to thank Zahra Pour-Safavi for her technical support in flowcytometery experiment and Azam Dalman for preparing the handmade glass pipettes. The authors declare no conflict of interest in this study.

Authors' Contributions

M.Z., E.A.; Performed all *in vitro* experiments, analyzed the data and wrote the manuscript. M.H.A.; Performed *in vivo* experiment and analysed the *in vivo* data. M.E.; Contributed to concept and design, financial support, and final approval of the manuscript. All authors read and approved the final manuscript.

References

- 1. Eaves CJ. Hematopoietic stem cells: concepts, definitions, and the new. Blood. 2015; 125(17): 2605-2613.
- Chabannon C, Kuball J, Bondanza A, Dazzi F, Pedrazzoli P, Toubert A, et al. Hematopoietic stem cell transplantation in its 60s: A platform for cellular therapies. Sci Transl Med. 2018; 10(436). pii: eaap9630.
- Patel SA, Rameshwar P. Stem cell transplantation for hematological malignancies: prospects for personalized medicine and cotherapy with mesenchymal stem cells. Curr Pharmacogenomics Person Med. 2011; 9(3): 229-239.
- Hough R, Danby R, Russell N, Marks D, Veys P, Shaw B, et al. Recommendations for a standard UK approach to incorporating umbilical cord blood into clinical transplantation practice: an update on cord blood unit selection, donor selection algorithms and conditioning protocols. Br J Haematol. 2016; 172(3): 360-370.
- Pineault N, Abu-Khader A. Advances in umbilical cord blood stem cell expansion and clinical translation. Exp Hematol. 2015; 43(7): 498-513.
- Walsh NC, Kenney LL, Jangalwe S, Aryee KE, Greiner DL, Brehm MA, et al. Humanized mouse models of clinical disease. Annu Rev Pathol. 2017; 12: 187-215.
- Tanner A, Taylor SE, Decottignies W, Berges BK. Humanized mice as a model to study human hematopoietic stem cell transplantation. Stem Cells Dev. 2014; 23(1): 76-82.
- Brehm MA, Shultz LD, Luban J, Greiner DL. Overcoming current limitations in humanized mouse research. J Infect Dis. 2013; 208 Suppl 2: S125-S130.
- Shultz LD, Brehm MA, Garcia JV, Greiner DL. Humanized mice for immune system investigation: progress, promise and challenges. Nat Rev Immunol. 2012; 12(11): 786-798.
- Kim AG, Vrecenak JD, Boelig MM, Eissenberg L, Rettig MP, Riley JS, et al. Enhanced in utero allogeneic engraftment in mice after mobilizing fetal HSCs by α4β1/7 inhibition. Blood. 2016; 128(20): 2457-2461.
- 11. Omori F, Lutzko C, Abrams-Ogg A, Lau K, Gartley C, Dobson H,

et al. Adoptive transfer of genetically modified human hematopoietic stem cells into preimmune canine fetuses. Exp Hematol. 1999; 27(2): 242-249.

- Fujiki Y, Fukawa K, Kameyama K, Kudo O, Onodera M, Nakamura Y, et al. Successful multilineage engraftment of human cord blood cells in pigs after in utero transplantation. Transplantation. 2003; 75(7): 916-922.
- Mychaliska GB, Rice HE, Tarantal AF, Stock PG, Capper J, Garovoy MR, et al. In utero hematopoietic stem cell transplants prolong survival of postnatal kidney transplantation in monkeys. J Pediatr Surg. 1997; 32(7): 976-981.
- Goodrich AD, Varain NM, Jeanblanc CM, Colon DM, Kim J, Zanjani ED, et al. Influence of a dual-injection regimen, plerixafor and CXCR4 on in utero hematopoietic stem cell transplantation and engraftment with use of the sheep model. Cytotherapy. 2014; 16(9): 1280-1293.
- Abe T, Masuda S, Ban H, Hayashi S, Ueda Y, Inoue M, et al. Ex vivo expansion of human HSCs with Sendai virus vector expressing HoxB4 assessed by sheep in utero transplantation. Exp Hematol. 2011; 39(1): 47-54.
- Abe T, Masuda S, Tanaka Y, Nitta S, Kitano Y, Hayashi S, et al. Maternal administration of busulfan before in utero transplantation of human hematopoietic stem cells enhances engraftments in sheep. Exp Hematol. 2012; 40(6): 436-444.
- McClain LE, Flake AW. In utero stem cell transplantation and gene therapy: Recent progress and the potential for clinical application. Best Pract Res Clin Obstet Gynaecol. 2016; 31: 88-98.
- Chowdhury N, Asakura A. In utero stem cell transplantation: potential therapeutic application for muscle diseases. Stem Cells Int. 2017; 2017: 3027520.
- Almeida-Porada G, Atala A, Porada CD. In utero stem cell transplantation and gene therapy: rationale, history, and recent advances toward clinical application. Mol Ther Methods Clin Dev. 2016; 5: 16020.
- Mao P, Xu L, Mo WJ, Yin Y, Xu YL, Lin XM. Effect of different cytokine combinations on the expression of CD49d and CXCR4 and ex vivo expansion of umbilical cord blood mononuclear cells. Zhongguo Shi Yan Xue Ye Xue Za Zhi. 2006; 14(2): 318-321.
- Medvinsky A, Rybtsov S, Taoudi S. Embryonic origin of the adult hematopoietic system: advances and questions. Development. 2011; 138(6): 1017-1031.
- Rice HE, Hedrick MH, Flake AW. In utero transplantation of rat hematopoietic stem cells induces xenogeneic chimerism in mice. Transplant Proc. 1994; 26(1): 126-128.
- Carrier E, Lee TH, Busch MP, Cowan MJ. Induction of tolerance in nondefective mice after in utero transplantation of major histocompatibility complex-mismatched fetal hematopoietic stem cells. Blood. 1995; 86(12): 4681-4690.
- Kim HB, Shaaban AF, Yang EY, Liechty KW, Flake AW. Microchimerism and tolerance after in utero bone marrow transplantation in mice. J Surg Res. 1998; 77(1): 1-5.
- Pallavicini MG, Flake AW, Madden D, Bethel C, Duncan B, Gonzalgo ML, et al. Hemopoietic chimerism in rodents transplanted in utero with fetal human hemopoietic cells. Transplant Proc. 1992;

24(2): 542-543.

- Turner CW, Archer DR, Wong J, Yeager AM, Fleming WH. In utero transplantation of human fetal haemopoietic cells in NOD/SCID mice. Br J Haematol. 1998; 103(2): 326-334.
- McNiece IK, Almeida-Porada G, Shpall EJ, Zanjani E. Ex vivo expanded cord blood cells provide rapid engraftment in fetal sheep but lack long-term engrafting potential. Exp Hematol. 2002; 30(6): 612-616.
- Young AJ, Holzgreve W, Dudler L, Schoeberlein A, Surbek DV. Engraftment of human cord blood-derived stem cells in preimmune ovine fetuses after ultrasound-guided in utero transplantation. Am J Obstet Gynecol. 2003; 189(3): 698-701.
- Blakemore K, Hattenburg C, Stetten G, Berg K, South S, Murphy K, et al. In utero hematopoietic stem cell transplantation with haploidentical donor adult bone marrow in a canine model. Am J Obstet Gynecol. 2004; 190(4): 960-973.
- Tanaka Y, Masuda S, Abe T, Hayashi S, Kitano Y, Nagao Y, et al. Intravascular route is not superior to an intraperitoneal route for in utero transplantation of human hematopoietic stem cells and engraftment in sheep. Transplantation. 2010; 90(4): 462-463.
- Rio P, Martinez-Palacio J, Ramirez A, Bueren JA, Segovia JC. Efficient engraftment of in utero transplanted mice with retrovirally transduced hematopoietic stem cells. Gene Ther. 2005; 12(4): 358-63.
- Ishii M, Matsuoka Y, Sasaki Y, Nakatsuka R, Takahashi M, Nakamoto T, et al. Development of a high-resolution purification method for precise functional characterization of primitive human cord blood-derived CD34-negative SCID-repopulating cells. Exp Hematol. 2011; 39(2): 203-213. e1.
- Noia G, Ligato MS, Cesari E, Visconti D, Fortunato G, Tintoni M, et al. Source of cell injected is a critical factors for short and long engraftment in xeno-transplantation. Cell Prolif. 2008; 41 Suppl 1: 41-50.
- Skopal-Chase JL, Pixley JS, Torabi A, Cenariu MC, Bhat A, Thain DS, et al. Immune ontogeny and engraftment receptivity in the sheep fetus. Fetal Diagn Ther. 2009; 25(1): 102-110.
- Troeger C, Surbek D, Schoberlein A, Schaft S, Dudler L, Hahn S, et al. In utero haematopoietic stem cell transplantation. Experiences in mice, sheep and humans. Swiss Med Wkly. 2007; 137 Suppl 155: 14S-19S.
- McNiece I, Jones R, Bearman SI, Cagnoni P, Nieto Y, Franklin W, et al. Ex vivo expanded peripheral blood progenitor cells provide rapid neutrophil recovery after high-dose chemotherapy in patients with breast cancer. Blood. 2000; 96(9): 3001-3007.
- Chen X, Gong XL, Katsumata M, Zeng YT, Huang SZ, Zeng F. Hematopoietic stem cell engraftment by early-stage in utero transplantation in a mouse model. Exp Mol Pathol. 2009; 87(3): 173-177.
- Zarrabi M, Afzal E, Asghari MH, Mohammad M, Es HA, Ebrahimi M. Inhibition of MEK/ERK signalling pathway promotes erythroid differentiation and reduces HSCs engraftment in ex vivo expanded haematopoietic stem cells. J Cell Mol Med. 2018; 22(3): 1464-1474.

Autologous Bone Marrow Stem Cell Transplantation in Liver Cirrhosis after Correcting Nutritional Anomalies, A Controlled Clinical Study

Abbas Esmaeilzadeh, M.D.^{1#}, Homeira Ommati, M.D.^{1#}, Mohammad Mahdi Kooshyar, M.D.², Lida Jarahi, M.D.³, Kambiz Akhavan Rezayat, M.D.¹, Samaneh Saberi, M.Sc.⁴, Massoud Vosough, M.D., Ph.D.^{5, 6*}, Ali Ghassemi, M.D.^{7*}

1. Department of Internal Medicine, Faculty of Medicine, Mashhad University of Medical Sciences, Gastroenterology and Hepatology Research Center, Mashhad, Iran

2. Department of Hematology and Oncology, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran 3. Department of Community Medicine, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

4. HPGC Group, Medical Biotechnology Department, Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran 5. Department of Regenerative Biomedicine, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

6. Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

7. Department of Pediatric Hematology and Oncology, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

#The first two authors equally contributed to this work.

*Corresponding Address: P.O.Box: 16635-148, Department of Regenerative Medicine, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

P.O.Box: 9177899191, Department of Pediatric Hematology and Oncology, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

Emails: masvos@royaninstitute.org, Ghasemial@mums.ac.ir

Received: 17/June/2018, Accepted: 8/October/2018 Abstract

Objective: Liver transplantation is the gold standard approach for decompensated liver cirrhosis. In recent years, stem cell therapy has raised hopes that adjusting some clinical and laboratory parameters could lead to successful treatments for this disease. Cirrhotic patients may have multiple systemic abnormalities in peripheral blood and irregular cell populations in bone marrow (BM). Correcting these abnormalities before BM aspiration may improve the effectiveness of cell-based therapy of liver cirrhosis.

Materials and Methods: In this controlled clinical trial study, 20 patients with decompensated liver cirrhosis were enrolled. Patients were randomly assigned to control and experimental groups. Blood samples were obtained to measure vitamin B12, folate, serum iron, total iron bonding capacity (TIBC) and ferritin before any intervention. Furthermore, the iron storage and fibrosis level in BM biopsies, as well as the percentage of different cell populations, were evaluated. Prior to cell isolation for transplantation, we performed palliative supplement therapy followed by a correction of nutritional deficiencies. Mononuclear cells (MNCs) were then isolated from BM aspirates and transfused through peripheral vein in patients in the experimental group. The model of end-stage liver disease (MELD) score, The international normalized ratio (INR), serum albumin and bilirubin levels were assessed at 0 (baseline), 3 and 6 months after cell transplantation.

Results: The MELD score (P=0.0001), INR (P=0.012), bilirubin (P<0.0001) and total albumin (P<0.0001) levels improved significantly in the experimental group after cell transplantation compared to the baseline and control groups. Moreover, the increase in serum albumin levels of patients in the experimental group was statistically significant 6 months after transplantation.

Conclusion: We have successfully improved the conditions of preparing -BM-derived stem cells for transplantation. Although these cells are relatively safe and have been shown to improve some clinical signs and symptoms temporarily, there need to be more basic studies regarding the preparation steps for effective clinical use (Registration number: IRCT2014091919217N1).

Keywords: Bone Marrow Stem Cells, Cell Therapy, Cirrhosis, Regenerative Medicine

Cell Journal (Yakhteh), Vol 21, No 3, October-December (Autumn) 2019, Pages: 268-273

Citation: Esmaeilzadeh A, Ommati H, Kooshyar MM, Jarahi L, Akhavan Rezayat K, Saberi S, Vosough M, Ghassemi A. Autologous bone marrow stem cell transplantation in liver cirrhosis after correcting nutritional anomalies, a controlled clinical study. Cell J. 2019; 21(3): 268-273. doi: 10.22074/cellj.2019.6108.

Introduction

Liver cirrhosis is one of the most common causes of death in the world and imposes huge financial burden (1, 2). Currently, the only available treatment for decompensated liver cirrhosis is orthotopic liver transplantation (OLT), which is limited to parameters such as the number of donated organs from cadavers or living donors, the high costs associated with both the procedure and the followup care, as well as post operation complications due to lifelong immunosuppression (3). However, recently researchers have focused on safe alternative possibilities to restore liver mass and function through stem cell therapy (4-7).

In 1999, Petersen et al. (8) showed that hematopoietic stem cells can contribute to liver regeneration. In 2000, Theise et al. (9) reported that hematopoietic stem cells successfully transformed into hepatocytes and cholangiocytes. They found Y-chromosome-positive hepatocyte-like cells in the liver of female recipients who had received male BM stem cells. Likewise, in another study in 2002, researchers showed that hematopoietic stem cells in both peripheral blood and BM could differentiate into hepatocytes and other epithelial cells (10). Since 1956, various hematologic disorders have been treated by bone marrow (BM) transplantation and different related clinical studies have been carried out using BM transplantation (11). Given these findings, hematopoietic stem cells may be a promising source for cell therapy in liver cirrhosis. In addition, various hematologic abnormalities, such as nutritional deficiencies, are secondary to liver cirrhosis and directly affect the population of BM-derived cells (12, 13).

To the best of our knowledge, at this point there is no study that has evaluated the efficacy of hematopoietic stem cell transplantation after correcting nutritional abnormalities in cirrhotic patients. Therefore, this clinical study was conducted to evaluate the efficiency of BM-mononuclear cell (MNC) transplantation through peripheral vein in cirrhotic patients after supplement treatment. Following cell transplantation, we also assessed whether the improved cells enhanced the results of stem cell therapy in cirrhotic patients.

In studies of liver diseases, a commonly used value is the model for end-stage liver disease (MELD), which is a scoring system for quantifying the severity of chronic liver diseases. To predict the survival rate, MELD uses the patient's values for serum bilirubin, serum creatinine, and international normalized ratio (INR) for prothrombin time (PT). Because the result (in seconds) for a PT performed on a normal individual will vary according to the type of analytical system, INR has been developed to normalize the results.

In the present clinical trial, we show a significant decrease in patient MELD scores at 3 and 6 months' post-cell transplantation.

Methods and Materials

Patient criteria and treatment

This controlled clinical trial study conducted at Mashhad University of Medical Science. Twenty patients were admitted at the "Research Center of Transplantation", Mashhad, Iran, from September 2014 to June 2015. All patients were diagnosed with liver cirrhosis, based on clinical, laboratory, radiologic and endoscopic data, and were all on a waiting list for liver transplantation. All patients received their regular medical treatment during the study. The exclusion criteria were refractory ascites, positive HIV antibody, primary sclerosing cholangitis (PSC), hepatocellular carcinoma (HCC), and portal and/or hepatic vein thrombosis.

Patients were randomly distributed into two groups, the experimental (n=10) and the control groups (n=10). Randomization was performed to reduce any possible bias and to adjust the study arms. In BM aspiration, our preferred site was the posterior iliac crest. Only the experimental group received intravenous infusion of autologous BM-derived MNCs. All the infusions were performed using veins in upper extremity.

Prior to the infusion of autologous BM-derived stem cells, the serum levels of vitamin B12, folate, iron, TIBC and ferritin were measured in each participant. In addition, the BM aspirates were analyzed in terms of cell quantity and quality, the level of iron storage and fibrosis. All malnutrition abnormalities and deficiencies in serum levels of the mentioned components and the percentage of cellular fractions of BM aspirates were corrected using supplement therapy before the cell infusion. The experimental group received IV infusions of autologous BM-derived MNCs and the control group received only autologous cell-free serum. For each individual a total of 20 ml of the cell suspension or cell-free serum was infused gradually. The cell infusion performed at the baseline. The patients were admitted and examined at Shariati Hospital (Mashhad, Iran) one day before cell infusion.

The proposal of this study was reviewed and approved by the Ethics Committee of Mashhad University of Medical Sciences, and registered for clinical trial studies in Iranian Ministry of Health (MOH). The registration number is IRCT2014091919217N1. This study was conducted in accordance with the Declaration of Helsinki. All patients were provided with written informed consent.

Cell preparation and transplantation

The BM samples (140-200 ml/patient) were collected under local anesthesia and general sedation in an operating room under sterile conditions. All collected BM aspirates were filtered to remove any fat, bone, clot and other possible particles that could be collected in blood collection bags. The remaining MNCs were washed and counted and their viability was assessed using trypan blue dye exclusion method. The mean viability of the transplanted MNCs was more than (95 ± 3) % in all the infusions. The MNCs were suspended in autologous serum at the final volume of 20 ml. Finally, in each patient the general condition, vital signs and any transfusion-related reactions were monitored for six hours after cell infusion.

Long term follow-up

A gastroenterologist examined the patients at baseline (0), 3, and 6 months post-infusion. A blood analysis was requested at each visit as follows: complete blood count, serum albumin and total bilirubin, blood urea nitrogen, PT and INR. The MELD score was also measured accordingly. No adverse effects were detected during or after cell transfusion.

Statistical analysis

We have presented our data as mean \pm SD. A two-way ANOVA followed by Sidak's multiple comparisons test was performed using Prism graphpad version 6.00 for Mac OS X graphpad Software, La Jolla, California, USA. A P<0.05 was considered statistically significant. Furthermore, ANCOVA was used to evaluate the difference in means of control and experimental groups, considering time covariate effect.

Results

The demographic data of the patients

We had initially recruited a total of 34 patients, however,

14 patients were excluded according to the inclusion/ exclusion criteria and ultimately 20 of them enrolled in the study (female/male ratio of experimental and control groups were 1/9 and 2/8, respectively). The mean age of the patients was 45.2 years (28-58 years old) and 46 years (21-62 years old) in control and experimental groups, respectively. The etiology of cirrhosis in four (20%) patients were autoimmune hepatitis (AIH), nine patients (45%) had suffered from viral hepatitis, five (25%) had an unknown origin, one (5%) had PSC and one (5%) had Wilson's disease. The descriptive underlying etiologies of the disease and demographic data of the participating patients are listed in Table 1. All the patients with nutritional deficiencies received supplement therapy prior to enrollment in the study.

The initial peripheral blood testing and bone marrow aspiration results

The peripheral blood tests of the patients were performed before BM aspiration and cell infusion. Hemoglobin concentration of the patients ranged between 12 to 17 g/ dl, but one male patient had a mild anemia (Hb<12 g/dl). The folate level (5 to 20 ng/ml) and the B12 level (59 to 895 pg/ml) were normal in all patients except for two individuals, who had folate levels less than 4.9 ng/ml and B12 levels less than 59 ng/ml. The transferrin saturation was normal in all patients (18-47%). The minimum concentration of ferritin was 12.8 ng/dl and its maximum concentration was 542 ng/dl. The normal range for ferritin starts at 12 ng/ml.

BM analysis showed no high-grade fibrosis in the patients. Moreover, five individuals had mild to moderate megaloblastic changes, three patients had micronormoblastic changes and the remaining had normal cellular analysis reports.

The mean weight of patients was 67.5 ± 4.8 kg. The mean number of total nucleated cell counts (TNC) was $(8.46 \pm 2.56 \times 10^3/\mu$ l) that $(58.59 \pm 7.834)\%$ of them were polymorphonuclear cells (PMN) and $(41.41 \pm 7.834)\%$ of them were MNCs. The mean number of transfused cells was $(8.059 \pm 2.539 \times 10^6$ cells/kg). Table 2 represents the number of MNCs that were infused into the patients.

Table 1: Descriptive underlying etiologies of the cirrhosis and demographic data of patients in experimental and control groups						
		Experimental group			Control group)
Patients ID.	Age (Y)	Gender	Etiology	Age (Y)	Gender	Etiology
P1	30	Male	AIH	28	Male	AIH
P2	37	Male	Hepatitis B	45	Male	Cryptogenic
P3	56	Male	Hepatitis C	54	Female	Hepatitis B
P4	28	Female	AIH	44	Male	PSC
P5	21	Male	Cryptogenic	58	Male	Cryptogenic
P6	54	Male	Hepatitis C	39	Male	Hepatitis B
P7	62	Male	AIH	50	Female	Cryptogenic
P8	56	Male	Hepatitis B	47	Male	Hepatitis B
Р9	58	Male	Cryptogenic	58	Male	Wilson
P10	58	Male	Hepatitis B	29	Male	Hepatitis B

PSC; Primary sclerosing cholangitis and AIH; Autoimmune hepatitis.

Table 2: The number of transfused MNC for patients in grou	p 1
--	-----

Patients ID.	TNC (10 ³ /µl)	PMN (%)	MNC (%)	BW (kg)	Transfused MNC (10 ⁶ cells/kg)
P1	11.2	65.8	34.2	72	8.1
P2	12.6	56.3	43.7	67	12.3
P3	7.1	49.4	50.6	68	7.7
P4	9.7	63.5	36.5	65	8.3
P5	3.3	74.3	25.7	59	2.15
P6	8.4	56.5	43.5	67	8.05
P7	8.8	54.3	45.7	64	9.5
P8	6.9	61.1	38.9	75	7.2
Р9	7.3	48.3	51.7	73	7.8
P10	9.3	56.4	43.6	65	9.49

TNC; Total nucleated cell, PMN; Polymorphonuclear cell, MNC; Mononuclear cell, and BW; Body weight.

Model of end-stage liver disease score

At 6 months post-cell transfusion the MELD score decreased in the experimental group to 16.2 ± 2.82 , which is statistically significant compared to the control group 21 ± 3.29 (P=0.0001). In the experimental group, there was no significant difference between 0 (baseline) (17.80 \pm 1.81), 3 (15.60 \pm 2.17) and 6 months (16.20 \pm 2.82) after transplantation (Fig.1), whereas, compared to the control group, the MELD score improved significantly 6 months post-cell infusion. Next, the influence of time as the covariate on independent MELD score in control and experimental groups were checked by ANCOVA. There was a significant difference in MELDI mean score [F (4,54)=5.272, P=0.001] between the cases and controls, whilst adjusting for time. It can be seen that for cases and controls the effect size is small (0.281).



Fig.1: Changes in MELD scores between control and experimental groups at different time points (baseline, three and six months post-transplantation). ***; P≤0.001.

International normalized ratio

The INR score of the control group increased from the baseline to 2.0 ± 0.17 and 2.4 ± 0.4 in 3 and 6 month post-transplantation, respectively. However, the score in the experimental group decreased slightly during the 6 months, which was statistically significant in both 3 (1.6 \pm 0.24, P=0.012) and 6 (1.5 \pm 0.37, P<0.0001) months post-cell infusion (Fig.2). In the experimental group, the INR score at 6 months (1.5 \pm 0.37) was even significantly lower compared to its baseline level (1.9 \pm 0.31, P=0.01). Next, the influence of time as the covariate on INR in control and experimental groups were checked by ANCOVA. There wasn't a significant difference in INR mean score [F (4,54)=0.989, P=0.422] between the cases and controls, whilst adjusting for time.

Albumin

Serum albumin levels of the patients in control and

experimental groups were different at baseline (control, 4.45 ± 0.25 vs. experimental, 3.18 ± 0.36), which was statistically significant (P<0.0001) and this lowered the power of study. As shown in Figure 3, at 6 months after the treatment, the average serum albumin level of the experimental group increased significantly compared to the control group (3.99 ± 0.50 vs. 2.90 ± 0.55 , P<0.0001). Next, the influence of time as the covariate on independent Serum albumin level in control and experimental groups were checked by ANCOVA. There was a significant difference in Albumin mean Level [F (4,54)=25.454, P<0.0001] between the cases and controls, whilst adjusting for time. It can be seen that for cases and controls the effect size is moderate (0.653).



Fig.2: Changes in international normalized ratio (INR) between the control and experimental groups at different time points [0 (baseline), 3 and 6 months post-transplant]. *; $P \le 0.05$ and ****; $P \le 0.0001$.



Fig.3: Changes in albumin levels between the control and experimental groups at different time points [0 (baseline), 3, and 6 months post-transplant]. ****; $P \le 0.0001$.

The serum level of albumin in the experimental group increased at 3 months post-transplantation

 $(4.06 \pm 0.48, P<0.0001)$, but slightly decreased by the 6 months time point $(3.99 \pm 0.50, P=0.0003)$. At both time points, however, the overall increases were statistically significant in comparison with the baseline level $(3.18 \pm 0.36, Fig.4)$.

Bilirubin

Serum bilirubin of the control group patients increased sharply and reached up to 3.11 ± 0.24 at 6 months after serum (placebo) infusion, whereas in the experimental group we observed a significant decrease in bilirubin level $(2.12 \pm 0.25, P=0.0009)$, 3 months after cell infusion. The significant decrease in bilirubin was observed at 6 months $(2.14 \pm 0.66, P<0.0001)$ after cell infusion, which was statistically significant compared to the control group.

The decrease in bilirubin level in the experimental group at 3 (2.12 \pm 0.25, P=0.0014) and 6 (2.14 \pm 0.66, P=0.002) months post-transplantation were also statistically significant compared to the baseline levels (2.69 \pm 0.24, Fig.4). Next, the influence of time as the covariate on independent serum bilirubin level in control and experimental groups were checked by ANCOVA. There was a significant difference in bilirubin mean Level [F (4,54)=22.464, P<0.0001] between the cases and controls, whilst adjusting for time. It can be seen that for cases and controls the effect size is moderate (0.625).



Fig.4: Changes in bilirubin between control and experimental groups at different time points (0, 3 and 6 months post-transplantation). ***; $P \le 0.001$ and ****; $P \le 0.0001$.

Discussion

Based on this study, after nutritional supplement treatment, hematopoietic stem cells transplantation could transiently improve MELD score, serum albumin level, INR score, as well as bilirubin level in cirrhotic patients. BM stem cell infusion results in considerable improvements in different disorders in basic and clinical studies (9, 14-16), particularly in clinical trials for liver cirrhosis in human patients (17-29).

Due to the short follow up period after transplantation (6 months), the efficacy of MNC infusion for an extended time is not clear yet. A transient improvement in MELD score was observed in the experimental group, but it was not statistically significant, at 6 months compared to the previous time point (3 months) after cell infusion. Nonetheless, in some previous studies, the follow up period after stem cell transplantation in cirrhotic patients has been up to 30 months with improvements in liver function tests (21). Therefore, our MELD data may have been affected by the shorter follow up period in our study.

In another previous study, the authors observed that MNC transplantation resulted in transient improvements in liver function, but it did not lead to a complete reversal from an abnormal condition into normal liver physiologic conditions (21). Therefore, given the fact that the mortality rate in patients in organ waiting lists is high, BM-derived MNCs may potentially give the cirrhotic patients a higher chance of survival while waiting for a matching liver for OLT (30).

Based on the results of the BM study and the peripheral blood testing data in cirrhotic patients, there were obvious discordances between the two. Presumably, BM is affected by nutritional deficiency in earlier stages of cirrhosis.

Stem cell therapy has had a growing progress in many disabling diseases, worldwide. Nowadays, different hematologic and non-hematologic disorders may be treated using stem cell transplantation. However, there are many limitations and doubts in terms of effectiveness of stem cell transplantation in liver cirrhosis. Scientists are now working on novel therapies to overcome these challenges.

Conclusion

We have shown that transplantation of BM-derived stem cells, which is a relatively safe procedure, transiently improves some crucial parameters in cirrhotic patients after correcting nutritional anomalies. However, to have a better and clearer conclusion, there need to be more basic and clinical studies and randomized clinical trials with a higher number of subjects with perhaps uniform etiologies.

Acknowledgements

Hereby, we appreciate the helps of all colleagues in "Research center of Gastroenterology and Hepatology" at Mashhad University of Medical Science. This study was funded by Mashhad University of Medical Science and Health Services. The authors declare no conflict of interest in this study.

Authors' Contributions

A.E., H.O.; Conceived the study, performed, and

analyzed experiments. M.M.K., K.A.R.; Analyzed the results, wrote the first draft of manuscript and discussed the results and finally approved the manuscript. L.J., S.S.; Analyzed the data and wrote the draft. M.V., A.Gh.; Designed and analyzed experiments, discussed the results, wrote the manuscript and approved the manuscript. All authors read and approved the final manuscript.

References

- Akbari Sari A, Kazemi Karyani A, Alavian SM, Arab M, Rostami Gholmohamadi F, Rezaei S. The economic burden of liver cirrhosis in Iran: a cost of illness study. Iran J Public Health. 2015; 44(4): 512-521.
- Estes C, Razavi H, Loomba R, Younossi Z, Sanyal AJ. Modeling the epidemic of nonalcoholic fatty liver disease demonstrates an exponential increase in burden of disease. Hepatology. 2018; 67(1): 123-133.
- Tsochatzis EA, Bosch J, Burroughs AK. Liver cirrhosis. Lancet. 2014; 383(9930): 1749-1761.
- Vosough M, Moslem M, Pournasr B, Baharvand H. Cell-based therapeutics for liver disorders. Br Med Bull. 2011, 100: 157-172.
- Vosough M, Moossavi S, Mardpour S, Akhlaghpoor S, Azimian V, Jarughi N, et al. Repeated intraportal injection of mesenchymal stem cells in combination with pioglitazone in patients with compensated cirrhosis: a clinical report of two cases. Arch Iran Med. 2016; 19(2): 131-136.
- Mohamadnejad M, Vosough M, Moossavi S, Nikfam S, Mardpour S, Akhlaghpoor S, et al. Intraportal infusion of bone marrow mononuclear or CD133+ cells in patients with decompensated cirrhosis: a double-blind randomized controlled trial. Stem Cells Transl Med. 2016; 5(1): 87-94.
- Mohamadnejad M, Ashrafi M, Alimoghaddam K, Vosough M, Mardpour S, Azimian V, et al. Surveillance for hepatocellular carcinoma after autologous stem cell transplantation in cirrhosis. Middle East J Dig Dis. 2012; 4(3): 145-149.
- Petersen BE, Bowen WC, Patrene KD, Mars WM, Sullivan AK, Murase N, et al. Bone marrow as a potential source of hepatic oval cells. Science. 1999; 284(5417): 1168-1170.
- Theise ND, Nimmakayalu M, Gardner R, Illei PB, Morgan G, Teperman L, et al. Liver from bone marrow in humans. Hepatology. 2000; 32(1): 11-16.
- Körbling M, Katz RL, Khanna A ,Ruifrok AC, Rondon G, Albitar M, et al. Hepatocytes and epithelial cells of donor origin in recipients of peripheral-blood stem cells. N Engl J Med. 2002; 346(10): 738-746.
- Passweg JR, Baldomero H, Bader P, Bonini C, Cesaro S, Dreger P, et al. Hematopoietic stem cell transplantation in Europe 2014: more than 40 000 transplants annually. Bone Marrow Transplant. 2016; 51(6): 786-792.
- Gonzalez-Casas R, Jones EA, Moreno-Otero R. Spectrum of anemia associated with chronic liver disease. World J Gastroenterol. 2009; 15(37): 4653-4658.
- Herbert V, Zalusky R, Davidson CS. Correlation of folate deficiency with alcoholism and associated macrocytosis, anemia, and liver disease. Ann Intern Med. 1963; 58: 977-988.
- Daley GQ, Goodell MA, Snyder EY. Realistic prospects for stem cell therapeutics. Hematology Am Soc Hematol Educ Program. 2003: 398-418.
- Bittner RE, Schöfer C, Weipoltshammer K, Ivanova S, Streubel B, Hauser E, et al. Recruitment of bone-marrow-derived cells by skel-

etal and cardiac muscle in adult dystrophic mdx mice. Anat Embryol (Berl). 1999; 199(5): 391-396.

- Theise ND, Badve S, Saxena R, Henegariu O, Sell S, Crawford JM, et al. Derivation of hepatocytes from bone marrow cells in mice after radiation-induced myeloablation. Hepatology. 2000; 31(1): 235-240.
- Terai S, Ishikawa T, Omori K, Aoyama K, Marumoto Y, Urata Y, et al. Improved liver function in patients with liver cirrhosis after autologous bone marrow cell infusion therapy. Stem Cells. 2006; 24(10): 2292-2298.
- Mohamadnejad M, Alimoghaddam K, Bagheri M, Ashrafi M, Abdollahzadeh L, Akhlaghpoor S, et al. Randomized placebo-controlled trial of mesenchymal stem cell transplantation in decompensated cirrhosis. Liver Int. 2013; 33(10): 1490-1496.
- Mohamadnejad M, Alimoghaddam K, Mohyeddin-Bonab M, Bagheri M, Bashtar M, Ghanaati H, et al. Phase 1 trial of autologous bone marrow mesenchymal stem cell transplantation in patients with decompensated liver cirrhosis. Arch Iran Med. 2007; 10(4): 459-466.
- Salama H, Zekri AR, Bahnassy AA, Medhat E, Halim HA, Ahmed OS, et al. Autologous CD34+ and CD133+ stem cells transplantation in patients with end stage liver disease. World J Gastroenterol. 2010; 16(42): 5297-5305.
- Yannaki E, Anagnostopoulos A, Kapetanos D, Xagorari A, Iordanidis F, Batsis I, et al. Lasting amelioration in the clinical course of decompensated alcoholic cirrhosis with boost infusions of mobilized peripheral blood stem cells. Exp Hematol. 2006; 34(11): 1583-1587.
- Peng L, Xie Dy, Lin BL, Liu J, Zhu HP, Xie C, et al. Autologous bone marrow mesenchymal stem cell transplantation in liver failure patients caused by hepatitis B: short-term and long-term outcomes. Hepatology. 2011; 54(3): 820-828.
- Huang XL, Luo L, Luo LY, Xue H, Wei LL, Yao YT, et al. Clinical outcome of autologous hematopoietic stem cell infusion via hepatic artery or portal vein in patients with end-stage liver diseases. Chin Med Sci J. 2014; 29(1): 15-22.
- Kim JK, Park YN, Kim JS, Park MS, Paik YH, Seok JY, et al. Autologous bone marrow infusion activates the progenitor cell compartment in patients with advanced liver cirrhosis. Cell Transplant. 2010; 19(10): 1237-1246.
- AlAhmari LS, AlShenaifi JY, AlAnazi RA, Abdo AA. Autologous bone marrow-derived cells in the treatment of liver disease patients. Saudi J Gastroenterol. 2015; 21(1): 5-10.
- Yao Y, Luo L, Xue H, Zou H, Wang G, An Z, et al. Autologous peripheral blood CD34+ stem cells transplanted into 100 patients with advanced cirrhosis. Zhonghua Gan Zang Bing Za Zhi. 2014; 22(9): 667-670.
- 27. Khan AA, Parveen N, Mahaboob VS, Rajendraprasad A, Ravindraprakash HR, Venkateswarlu J, et al. Safety and efficacy of autologous bone marrow stem cell transplantation through hepatic artery for the treatment of chronic liver failure: a preliminary study. Transplant Proc. 2008; 40(4): 1140-1144.
- El-Ansary M, Abdel-Aziz I, Mogawer S, Abdel-Hamid S, Hammam O, Teaema S, et al. Phase II trial: undifferentiated versus differentiated autologous mesenchymal stem cells transplantation in Egyptian patients with HCV induced liver cirrhosis. Stem Cell Rev. 2012; 8(3): 972-981.
- Kharaziha P, Hellström PM, Noorinayer B, Farzaneh F, Aghajani K, Jafari F, et al. Improvement of liver function in liver cirrhosis patients after autologous mesenchymal stem cell injection: a phase I–II clinical trial. Eur J Gastroenterol Hepatol. 2009; 21(10): 1199-1205.
- Gaudio E, Carpino G, Cardinale V, Franchitto A, Onori P, Alvaro D. New insights into liver stem cells. Dig Liver Dis. 2009; 41(7): 455-462.

Uterine Dendritic Cells Modulation by Mesenchymal Stem Cells Provides A Protective Microenvironment at The Feto-Maternal Interface: Improved Pregnancy Outcome in Abortion-Prone Mice

Maryam Eskandarian, M.Sc., Seyed Mohammad Moazzeni, Ph.D.*

Department of Immunology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

*Corresponding Address: P.O.Box: 14115-331, Department of Immunology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

Email: Moazzenni@modares.ac.ir

Received: 22/July/2018, Accepted: 31/January/2019

Abstract

Objective: Dendritic cells (DCs) as major regulators of the immune response in the decidua play a pivotal role in establishment and maintenance of pregnancy. Immunological disorders are considered to be the main causes of unexplained recurrent spontaneous abortions (RSAs). Recently, we reported that mesenchymal stem cells (MSCs) therapy could improve fetal survival and reduce the abortion rate in abortion-prone mice, although the precise mechanisms of this action are poorly understood. Since MSCs have been shown to exert immunomodulatory effects on the immune cells, especially DCs, this study was performed to investigate the capability of MSCs to modulate the frequency, maturation state, and phenotype of uterine DCs (uDCs) as a potential mechanism for the improvement of pregnancy outcome.

Materials and Methods: In this experimental study, adipose-derived MSCs were intraperitoneally administered to abortion-prone pregnant mice on the fourth day of gestation. On the day 13.5 of pregnancy, after the determination of abortion rates, the frequency, phenotype, and maturation state of uDCs were analyzed using flow cytometry.

Results: Our results indicated that the administration of MSCs, at the implantation window, could significantly decrease the abortion rate and besides, increase the frequency of uDCs. MSCs administration also remarkably decreased the expression of DCs maturation markers (MHC-II, CD86, and CD40) on uDCs. However, we did not find any difference in the expression of CD11b on uDCs in MSCs-treated compared to control mice.

Conclusion: Regarding the mutual role of uDCs in establishment of a particular immunological state required for appropriate implantation, proper maternal immune responses and development of successful pregnancy, it seems that the modulation of uDCs by MSCs could be considered as one of the main mechanisms responsible for the positive effect of MSCs on treatment of RSA.

Keywords: Dendritic Cells, Mesenchymal Stem Cells, Spontaneous Abortion

Cell Journal(Yakhteh), Vol 21, No 3, October-December (Autumn) 2019, Pages: 274-280 _

Citation: Eskandarian M, Moazzeni SM. Uterine dendritic cells modulation by mesenchymal stem cells provides a protective microenvironment at the fetomaternal interface: improved pregnancy outcome in abortion-prone mice. Cell J. 2019; 21(3): 274-280. doi: 10.22074/cellj.2019.6239.

Introduction

In allogeneic pregnancy, despite the close contact of the maternal immune system and immunologically foreign fetal-placental alloantigens, the mother's immune system not only does not reject the fetus but also helps the fetus to implant and develop within the uterus (1). It is well established that in a normal pregnancy, maternal immune responses at the feto-maternal interface are precisely controlled by immunoregulatory mechanisms (1, 2). In contrast, failure in the immune response fine-tuning leads to disturbed pregnancy outcomes such as recurrent spontaneous abortion (RSA) and preeclampsia (2-4). The pattern of the immune cells and immunoregulatory mediators produced within the decidua play a crucial function in the maintenance of tolerance toward the semi-allogeneic fetus (5). Recently, immunological disorders are reported as the main players in the etiology of idiopathic RSA (6).

A wide range of immune cells such as natural killer cells (NK), macrophages (MQ), T lymphocytes, natural killer T cells (NKT), regulatory T cells (Tregs), and Dendritic cells (DCs) are present in the pregnant uteri (7).

Among these cells, uterine DCs (uDCs) are considered the major regulators of the immune responses, mainly present at the interface of the innate and acquired immune responses, adjusting T-cell mediated immunity and stimulating the induction of regulatory T-cells, etc. These immunoregulatory mechanisms collectively lead to tolerogenic microenvironment and protection of semiallogeneic embryo (8, 9). uDCs are not only crucial for the generation of maternal immunologic tolerance but also essential for the implantation of embryo via regulating stromal cell differentiation and vascular maturation and remodeling (10). It is supposed that decidual DCs may also play an important role in the etiology of RSA, and any disturbance in their distribution, maturation state, and function could affect the pregnancy outcome that may lead to a disturbed pregnancy (11).

It is well-established that the number, phenotype, and maturation state of DCs determine the tolerogenic or stimulatory nature of the immune response and its intensity (12). uDCs in a normal pregnancy usually have an immature phenotype and are functionally impaired in terms of immunogenic antigen presentation and T-cell activation (12, 13). In contrast, some functional changes in decidual DCs have been reported in pathological conditions such as RSAs and preeclampsia (14, 15). It is well-proven that the tissue environment (including cellular context and secreted factors) profoundly affects the maturity and function of DCs (16, 17). In other words, the behavior of uDCs is extremely controlled by the microenvironment in which they are developed (18). Therefore, it is supposed that the microenvironment of decidua can either foster DCs to promote cell toleration at the fetal-maternal interface or trigger an immune response that is associated with fetal rejection (12).

Regarding the importance of immune system failures, particularly dendritic cells (DCs) malfunctions in unexplained RSA, several therapeutic protocols based on immune modulation have been developed, including paternal leukocyte immunization, and aspirin, progesterone, and immunoglobulins administration. These treatments have yielded some promising results, although several controversial outcomes have also been reported (19). In recent years, the treatment of RSA using MSCs has been implicated due to their immunomodulatory properties, low immunogenicity, and ability to migrate to the site of inflammation prefrentially (20-22).

In our previous study, we showed that the administration of MSCs to an abortion-prone murine model (CBA/J×DBA/2) improved fetal survival and reduced the rate of abortion (20-22). Consequently, we demonstrated that MSCs could be a suitable potential candidate for the treatment of RSA. MSCs have been shown to exert immunomodulatory effects on immune cells, especially DCs. Recent studies have demonstrated a critical role for MSCs in the modulation of DCs differentiation, maturation, and function (23, 24).

In this study, we hypothesized that MSCs might exert their protective effects, at least in part, by modulating the context of the DC of uterine. Thus, the frequency, phenotype, and maturation stage of uDCs in abortion-prone pregnant mice following MSCs therapy were evaluated.

Material and Methods

Mice and mating

In this experimental study, female CBA/J (6-10 weeks old), male BALB/c, and male DBA/2 (8-10 weeks old) mice were obtained from the Pasteur Institute of Iran (Tehran, Iran), housed in an animal facility under optimal condition of temperature, humidity, and 12-hours light/ dark cycle. All animals were handled under procedures approved by the Ethical Committee of Tarbiat Modares University (IR.TMU.REC.1394.286).

Female CBA/J mice were allowed to mate with male DBA/2 or BALB/c mice by overnight cohabitation and then, checked for vaginal plug every morning. The day of vaginal plug observation was considered the day 0.5 of pregnancy.

The mating of female CBA/J mice with male DBA/2 was

determined as the immunogenic-abortion mouse model. 1×10^6 adipose-derived MSCs were intraperitoneally injected to DBA-mated CBA/J females at the implantation period (the day 4.5 of gestation) (MSCs-treated group, n=5). Female CBA/J mice in the control group (CBA/J×DBA/2) just received phosphate-buffered saline (PBS) at the implantation time (n=5). PBS-treated BALB/c-mated females (n=5) were used as the normal pregnant controls.

Mesenchymal stem cells isolation and characterization

MSCs were obtained from adipose tissue of CBA/J mice (5-7 weeks), as we described previously (20-22). Simply, abdominal fat tissue from non-pregnant CBA/J mice was cut into small pieces and carefully exposed and digested by collagenase type I (Gibco, Germany). The obtained cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, Germany). Non-adherent hematopoietic cells were removed after 18-24 hours and adherent cells were cultured to the second passage when the cells were used for the administration. MSCs were characterized through the evaluation of their expressed cell surface markers by flow cytometry (FACSCanto, BD, San Jose, CA, USA) and capability to differentiate into adipocytes and osteoblasts.

Pregnancy outcome and preparation of uterine cells

Pregnant mice were sacrificed on the day 13.5 of gestation, and their uteri horns were completely removed. The abortion rate was recorded as we described previously (11, 20). After complete removal of fetuses and placenta, the uteri were minced into small fragments and digested using 1mg/ml collagenase IV (Roch, Germany) and 0.2 mg/ml DNase (Sigma-Aldrich, St. Louis, USA). Digested tissue was then filtered through a 70 μ m strainer and washed twice in cold PBS. Finally, cells were collected and re-suspended in cold PBS.

Flow cytometry analysis

Single cells prepared from the uterus were treated with antibody against CD16/CD32 (anti-Fcy receptor III/ II antibody) to avoid non-specific antibody binding through Fc receptors blockage. Cells were then washed twice with ice-cold PBS (pH=7.2) and stained with PEconjugated hamster anti-mouse CD11c and one of the APC-conjugated monoclonal antibodies (anti-MHC-II, anti-CD86, anti-CD11b, and anti-CD40) and APC-Cy7-conjugated antibody (anti-CD45) (all antibodies obtained from eBioscience, San Diego, USA). Cells were subsequently analyzed by flow cytometry (FACSCanto II, BD, San Jose, CA, USA) and the obtained data were analyzed using the FlowJo software (version 6.07). The uterine cells were selected on dot plots of side and forward scatters. CD45-positive cells as uterine leukocytes were gated and the frequency of CD11c-positive cells (mouse uDCs) was evaluated in uterine leukocyte population (Fig.1). The expression of the DC lineage marker (CD11b) and co-stimulatory molecules (CD40, CD86, and MHC-II) were assessed on CD11c⁺ cells.



Fig.1: On the day 13.5 of gestation, single cell suspensions were prepared from uteri of MSCs-treated, MSCs-untreated and normal pregnant mice. The cells were stained with monoclonal antibodies against CD45 and CD11c and analyzed by flow cytometry. **A.** Representative dot plots were gated on forward versus side scatter (FSC/SSC) to determine uterine cells population, **B.** CD45⁺ cells were gated on selected uterine cells, and **C.** Than CD11c⁺ cells selected among the CD45⁺ cells to show the percentage of uDCs. MSCs; Mesenchymal stem cells and uDC; Uterine dendritic cells.

Statistical analysis

The differences between the groups were evaluated using a standard parametric test (One- way ANOVA test) followed by Turkey post hoc tests, after approval of the normal distribution of the obtained data by the Kolmogorov-Smirnov test. The results were considered statistically significant if the P was less than 0.05. The results were presented as the mean and standard deviation (mean \pm SD) of five separate experiments. All statistical analyses were performed using the Prism software (version 6.07).

Results

Characterization of mesenchymal stem cells

Flow cytometry analysis confirmed that MSCs strongly express typical markers such as Sca-1, CD90, CD105, CD29, and CD73 while they were negative for the expression of hematopoietic markers including CD45, CD34, CD11b, and MHC-II. The differentiation potency of MSCs into adipocyte was demonstrated by the observation of triglyceride-containing vacuoles in the cell cytoplasm, by oil red staining. Alizarin-red S staining of calcium accumulation also showed the osteogenic potential of MSCs (data not shown).

Effect of mesenchymal stem cells therapy on pregnancy outcome

In accordance with our previous results (20, 22) we found that MSCs administration during the implantation window remarkably decreased the abortion rate in the abortion-prone mouse model. A statistically significant lower abortion rate was shown in the MSCs-treated mice compared with the control group (6.5 ± 6.08 vs. 34.6 ± 7.7 , P<0.001). The abortion rate in the normal pregnant group was 5.3 ± 4.3 .

Effect of mesenchymal stem cells treatment on uterine dendritic cells

The flow cytometry analysis demonstrated that the average density of uDCs was significantly higher in the MSCs-treated mice compared with the control group ($12.3 \pm 1.5\%$ vs. $6.5 \pm 1.3\%$, P<0.0001, Fig.2). Notably, we observed that the mean frequency of uDCs in the MSCs-treated group was similar to normal pregnant control mice ($12.3 \pm 1.5\%$ vs. 11.2 ± 1.2 , P=0.3, Fig.2). Meanwhile, we found that the average percentage of uDCs in the control group (abortion-prone mice) was noticeably lower than the normal pregnant and MSCs-treated groups ($6.5 \pm 1.3\%$ vs. $11.2 \pm 1.2\%$, P<0.001) and ($6.5 \pm 1.3\%$ vs. $12.3 \pm 1.5\%$, P<0.0001) respectively (Fig.2).

Further analysis showed that the expression of CD86, CD40, and MHC-II markers on the uDCs in control group (abortion-prone mice) ($39.2 \pm 2.8\%$, $27.6 \pm 2.9\%$, and $62.5 \pm 2.1\%$ respectively) was noticeably higher than normal pregnant group ($16.5 \pm 2.5\%$, $5.7 \pm 1.7\%$, and $45.6 \pm 3.2\%$, respectively, P<0.001, P<0.001, P<0.001, Fig.3). MSCs administration caused a significant decrease in the expression of the early-mentioned co-stimulatory molecules on uDCs of MSCs-treated mice ($24.3 \pm 2.2\%$, $10.7 \pm 2.1\%$, $47.7 \pm 2.5\%$) compared with the control group (P<0.001, P<0.001, P<0.001, Fig.3).

Further investigations indicated that the relative percentage of CD11b⁺ uDCs in normal pregnant mice (92.5 \pm 3.5%) was higher than the control group (abortion-prone group) (72.6 \pm 4.8%, P<0.0001). Treatment with MSCs did not change the frequency of CD11b-positive cells in uterus compared with the control group (75.3 \pm 4.5% vs. 72.6 \pm 4.8%, P=0.3, Fig.3).



Fig.2: The effect of MSCs administration on frequency of uDCs. **A.** The dot plots show the percentage of uDCs (CD11c⁺ cells) in MSCs-treated, untreated group (control-group) and normal pregnant groups. The plots are representative of five independent experiment in each group and **B.** The graph indicates that MSCs administration significantly increased the frequency of DCs in uterine. The differences between the groups were evaluated using a standard parametric test (one-way ANOVA test). The results show the mean ± SD of five independent experiments. ***, ****; P<0.001 and P<0.0001 respectively, MSCs; Mesenchymal stem cells, uDC; Uterine dendritic cells, and NS; Not significant.



Fig.3: The effect of MSCs administration on the immunophentype of uDCs. The uterine cells were isolated from uterine of MSC-treated, un-treated group (controlgroup) and normal pregnant (normal-pregnancy) mice at the gd 13.5, stained with monoclonal antibodies against CD45, CD11c and one of the monoclonal antibodies (anti-MHC-II, anti-CD86, anti-CD11b and anti-CD40) then analyzed by flow cytometry. **A.** The CD11c positive uDCs were selected from the CD45⁺ cells of whole uterine cell population. Then the expression of CD11b, CD86, CD40 and MHC-II on uDCs was evaluated. The red histograms show the isotype controls and **B.** The graphs indicate that MSCs administration significantly decreased the expression of MHC-II and co-stimulatory molecules (CD86, CD40) on uDCs while CD11b⁺ DCs were not changed following MSC therapy. The differences between the groups were evaluated using a standard parametric test (one-way ANOVA test). The results show the mean ± SD of five independent experiments. **, ***, and ****; P<0.01, P<0.001, and P<0.0001 respectively, MSCs; Mesenchymal stem cells, uDC; Uterine dendritic cells, and NS; Not significant.

Discussion

Because of immunosuppressive properties of MSCs, they display therapeutic efficacy for the treatment of various immune-related diseases such as inflammatory, auto-immune and graft-versus-host (GVH) diseases (23). Many studies have reported that MSCs can diminish the clinical relapse rate in GVHD and ameliorate the function of defective organs in autoimmune disease models (25). Moreover, MSCs transplantation was shown to be safe due to their low immunogenicity (23). However, there are some limitations in the use of stem cells for cell therapy such as the potential malignancy development, finite replicative lifespan, ethical consideration, and the probability of somatic mutation. However, these disadvantages are most common in the case of using embryonic stem cells and induced pluripotent stem cells (iPSCs) not MSCs (26). In our previous studies (20-22), we also showed that MSCs therapy could improve fetal survival and reduce the abortion rate in abortionprone mice. Many studies have reported that fetal death in this model is related to the aberrant immune response including malfunction of NK cells and MQ, increment of Th1 cytokines, and the reduction of regulatory T cells frequency (27). Understanding the precise mechanisms accounting for the positive effect of MSCs on reducing the abortion rate in abortion-prone mice seems to be crucial. Based on the importance of uDCs in the induction of specific tolerogenic state required for proper maternal immune responses and the establishment of successful pregnancy, we investigated whether MSCs are capable of regulating uDCs recruitment and maturation state during gestation and finally improving pregnancy outcome.

Our results showed that uDCs are significantly less frequent in the uteri of abortion-prone mice compared with the normal pregnant animals. MSCs-therapy caused a significant upregulation in the frequency of uDCs which came close to the normal pregnancy.

It is well-defined that uDCs play a crucial role in the maintenance and development of pregnancy as the activators and regulators of T-cell immunity (12). uDCs are not only essential for the induction of tolerogenic responses against the semi-allogeneic embryo but also play an important role in uterine receptivity and vascular maturation during the implantation of the embryo (10). Fine-balance of uDCs frequency is crucial for the establishment and development of a successful pregnancy. In agreement with this idea, Krey et al. (28) reported that the depletion of uDCs before the implantation leads to pregnancy failure due to disturbed embryo implantation and decidualization. Also, according to Tirado-González et al. (15) the number of decidual DC-SIGN+ cells in human RSA cases were considerably decreased compared with the normal pregnancies. Furthermore, it was shown that the administration of syngeneic DCs to an abortionprone murine model reduces the rate of abortion, yet the mechanism underlying this function is poorly understood (29).

It was shown that MSCs exert immunomodulatory effects on immune cells (especially DCs) through the secretion of various components, as well as a direct cellcell contact (23). Numerous in vitro studies demonstrated that MSCs suppress the generation of myeloid DCs from both monocytes and CD34⁺ cell precursors. However, the immunosuppressive effect of MSCs is related to their surrounding microenvironment, which plays a decisive role in determining their function (30). It is now known that inflammatory cytokines such as IFN- γ and TNF- α augment the immunomodulatory roles of MSCs (20, 31). A large body of research has reported the dominance of inflammatory responses in the decidua, at the beginning of pregnancy and during the implantation period, when we also have administered the MSCs (1). This inflammatory situation not only helps the attraction and migration of MSCs to the uterine but also enhances their immunomodulatory effects (31, 32).

Of note, MSCs produce several cytokines and chemokines, including colony stimulating factor (CSF-1), granulocyte-monocyte colony stimulating factor (GM-CSF), IL-8, and CCL2, playing major roles in recruiting the immune cells, particularly DCs within the uterus (23). It was shown that GM-CSF could promote DC differentiation *in vitro*, as well as enhancing DC expansion *in vivo* (33). MSCs may also regulate the trafficking of immune cells (especially uDCs) toward the endometrium through modulating the secretion of GM-CSF by uterine epithelial cells. Tremellen et al. (34) demonstrated that GM-CSF synthesis is upregulated in uterine epithelial cells by seminal factors, especially TGF- β . TGF- β is also among the most important cytokines secreted by MSCs (23).

Moreover, our findings showed that uDCs in MSCsuntreated abortion-prone mice were more mature compared with the normal pregnant mice. MSCs therapy dramatically decreased the expression of MHC-II and co-stimulatory molecules (CD86, CD40) on uDCs. It is believed that the maturation stage of uDCs also plays an essential role in the etiology of RSA (12). uDCs in normal pregnancy are mostly immature and inefficient for the induction of immunogenic T-cells response (13). Consistent with this idea, Blois et al. proposed that the increased number of mature uDCs might be associated with a high rate of abortion in CBA/J×DBA/2 mating (12). Also, Askelund et al. (35) showed that, at 8 weeks of gestation, mature (CD83⁺) uDCs were significantly more frequent in women with RSA than the normal controls. It seems that these abnormally high immunogenic uDCs can prevail the tolerance to the fetal alloantigens and eventually lead to fetal rejection (12). There is a substantial body of evidence from in vitro studies revealing that MSCs can decrease the expression of MHC-II and co-stimulatory molecules on DCs (23, 36). MSCs secrete critical mediators such as IL-10, TGF- β 1, and PGE2, which are major regulators of DCs (23). This immunoregulatory factors prevent the maturation of DCs and induce tolerogenic DCs that are essential for a normal pregnancy (37, 38).

Several studies demonstrated the beneficial effects of stem cell-based therapy on the treatment of inflammatory and autoimmune diseases through the upregulation of anti-inflammatory cytokines, and remarkable reduction in the expression of pro-inflammatory cytokines (39, 40). In our previous studies, we also reported that MSCs therapy in abortion-prone mice could modulate the pattern of inflammatory and anti-inflammatory cytokines (20, 21). Regarding the substantial role of immunoregulatory cytokines (especially IL-10 and TGF- β) in modulating the phenotype and maturation stage of uDCs at the feto-maternal interface (12, 13), it seems that increased release of these cytokines following MSCs-therapy could be taken into account as one of the major mechanisms responsible for the induction of tolerogenic DCs.

Conclusion

Collectively, our results propose that MSCs therapy can normalize the frequency and maturation state of uDCs in abortion-prone mice. Since, the deregulated immune response is known to be the central player in the etiology of abortion in this model and accepted immunomodulatory effects of MSCs on immune cells especially DCs, as well as considering the key role of uDCs in the induction of tolerogenic response to fetal alloantigens and the development of normal pregnancy, it seems that the modulation of uDCs by MSCs could be one of the primary mechanisms accounting for the positive effect of MSCs in RSA therapy.

Acknowledgments

The authors would like to acknowledge financial support from Tarbiat Modares University and National Institute for Medical Research Development (NIMAD), Tehran, Iran. The authors report no declarations of interest.

Authors' Contributions

M.E., S.M.M.; Both contributed to the conception and design of the study, as well as the interpretation of the obtained data. M.E.; Did all the experimental work, data collection, and statistical analysis. S.M.M.; Is responsible for overall supervision. All authors have read and approved the final manuscript.

References

- 1. Mor G, Aldo P, Alvero AB. The unique immunological and microbial aspects of pregnancy. Nat Rev Immunol. 2017; 17(8): 469-482.
- Trowsdale J, Betz AG. Mother's little helpers: mechanisms of maternal-fetal tolerance. Nat Immunol. 2006; 7(3): 241-246.
- 3. Erlebacher A. Immunology of the maternal-fetal interface. Annu Rev Immunol. 2013; 31: 387-411.
- Aghaeepour N, Ganio EA, Mcilwain D, Tsai AS, Tingle M, Van Gassen S, et al. An immune clock of human pregnancy. Sci Immunol. 2017; 2(15). pii: eaan29465.
- Sanguansermsri D, Pongcharoen S. Pregnancy immunology: decidual immune cells. Asian Pac J Allergy Immunol. 2008; 26(2-3): 171-181.
- Beaman KD, Ntrivalas E, Mallers TM, Jaiswal MK, Kwak-Kim J, Gilman-Sachs A. Immune etiology of recurrent pregnancy loss and its diagnosis. Am J Reprod Immunol. 2012; 67(4): 319-325.
- 7. PrabhuDas M, Bonney E, Caron K, Dey S, Erlebacher A, Fazlea-

bas A, et al. Immune mechanisms at the maternal-fetal interface: perspectives and challenges. Nat Immunol. 2015; 16(4): 328-334.

- Mofazzal MA, Karimi M, Azadmanesh K, Hassan ZM, Moaazeni SM. The effect of chitosan-tripolyphosphate nanoparticles on maturation and function of dendritic cells. Comp Clin Pathol. 2014; 23: 1421-1427.
- Shah NM, Herasimtschuk AA, Boasso A, Benlahrech A, Fuchs D, Imami N, et al. Changes in T cell and dendritic cell phenotype from mid to late pregnancy are indicative of a shift from immune tollerance to immune activation. Front Immunol. 2017; 8: 1138.
- Plaks V, Birnberg T, Berkutzki T, Sela S, BenYashar A, Kalchenko V, et al. Uterine DCs are crucial for decidua formation during implantation in mice. J Clin Invest. 2008; 118(12): 3954-3965.
- Ahmadabad HN, Salehnia M, Saito S, Moazzeni SM. Decidual soluble factors, through modulation of dendritic cells functions, determine the immune response patterns at the feto-maternal interface. J Reprod Immunol. 2016; 114: 10-17.
- Blois SM, Kammerer U, Alba Soto C, Tometten MC, Shaikly V, Barrientos G, et al. Dendritic cells: key to fetal tolerance? Biol Reprod. 2007; 77(4): 590-598.
- Laskarin G, Kammerer U, Rukavina D, Thomson AW, Fermendez N, Blois SM. Antigen- presenting cells and materno-fetal tolerance: an emerging role for dendritic cells. Am J Reprod Immunol. 2007; 58(3): 255-267.
- Darmochwal-Kolarz D, Rolinski J, Tabarkiewicz J, Leszczynska-Gorzelak B, Buczkowski J, Wojas K, et al. Myeloid and lymphoid dendritic cells in normal pregnancy and pre-eclampsia. Clin Exp Immunol. 2003; 132(2): 339-344.
- Tirado-González I, Muñoz-Fernández R, Blanco O, Leno-Durán E, Abadía-Molina AC, Olivares EG. Reduced proportion of decidual DC-SIGN+ cells in human spontaneous abortion. Placenta. 2010; 31(11): 1019-1022.
- Fricke I, Gabrilovich DI. Dendritic cells and tumor microenvironment: a dangerous liaison. Immunol Invest. 2006; 35(3-4): 459-483.
- Shao Q, Ning H, Lv J, Liu Y, Zhao X, Ren G, et al. Regulation of Th1/Th2 polarization by tissue inhibitor of metalloproteinase-3 via modulating dendritic cells. Blood. 2012; 119(20): 4636-4644.
- Torabi-Rahvar M, Bozorgmehr M, Jeddi-Tehrani M, Zarnani A. Potentiation strategies of dendritic cell based antitumor vaccines: combinational therapy takes the front seat. Drug Discov Today. 2011; 16(15-16): 733-740.
- Wong LF, Porter TF, Scott JR. Immunotherapy for recurrent miscarriage. Cochrane Database Syst Rev. 2014; (10): CD000112.
- Sadighi-Moghaddam B, Salek-Farrokhi A, Namdar-Ahmadabad H, Barati M, Moazzeni SM. Mesenchymal stem cell therapy prevents abortion in CBA/J XDBA/2 mating. Reprod Sci. 2018; 25(8): 1261-1269.
- Salek-Farokhi A, Zarnani AH, Moazzeni SM. Mesenchymal stem cells therapy protects fetuses from resorption and induces Th2 type cytokines profile in abortion prone mouse model. Transpl Immunol. 2017; 47: 26-31.
- Rezaei F, Moazzeni SM. Comparison of the therapeutic effect of syngeneic, allogeneic, and xenogeneic adipose tissue-derived mesenchymal stem cells on abortion rates in a mouse model. Cell J. 2019; 21(1): 92-98.
- Ma S, Xie N, Li W, Yuan B, Shi Y, Wang Y. Immunobiology of mesenchymal stem cells. Cell Death Differ. 2014; 21(2): 216-225.
- Bozorgmehr M, Zarnani AH, Sheikhian A, Salehnia M, Jabbari Arfaee A, Moazzeni SM. Inhibitory effect of menstrual blood stromal stem cells on generation of dendritic cells from peripheral blood monocyte. MJMS. 2012; 14(4): 23-37.
- Zhang J, Huang X, Wang H, Liu X, Zhang T, Wang Y, et al. The challenges and promises of allogeneic mesenchymal stem cells for use as a cell-based therapy. Stem Cell Res Ther. 2015; 6: 234.
- Rusu E, Necula LG, Neagu A, Alecu M, Stan C, Albulescu R, et al. Current status of stem cell therapy: opportunities and limitations. Turk J Biol. 2016; 40: 955-967.
- Kwak-Kim J, Park JC, Ahn HK, Kim JW, Gilman-Sachs A. Immunological modes of pregnancy loss. Am J Reprod Immunol. 2010; 63(6): 611-623.
- Krey G, Frank P, Barrientos G, Cordo-Russo R, Ringel F, Moschansky P. In vivo dendritic cell depletion reduces breeding efficiency, affecting implantation and early placental development in mice. J Mol Med (Berl). 2008; 86(9): 999-1011.
- Miranda S, Litwin S, Barrientos G, Szereday L, Chuluyan E, Bartho JS, et al. Dendritic cells therapy confers a protective microenvironment in murine pregnancy. Scand J Immunol. 2006; 64(5): 493-499.

279

- El Omar R, Beroud J, Stoltz JF, Menu P, Velot E, Decot V, et al. Umbilical cord mesenchymal stem cells: the new gold standard for mesenchymal stem cell-based therapies? Tissue Eng Part B Rev. 2014; 20(5): 523-544.
- Wei X, Yang X, Han ZP, Qu FF, Shao L, Shi YF. Mesenchymal stem cells: a new trend for cell therapy. Acta Pharmacol Sin. 2013; 34(6): 747-754.
- Shi Y, Su J, Roberts A, Shou P, Rabson AB, Ren G. How mesenchymal stem cells interact with tissue immune responses. Trends Immunol. 2012; 33(3): 136-143.
- van de Laar L, Coffer PJ, Woltman AM. Regulation of dendritic cell development by GM-CSF: molecular control and implications for immune homeostasis and therapy. Blood. 2012; 119(15): 3383-3393.
- Tremellen KP, Seamark RF, Robertson SA. Seminal transforming growth factor beta1 stimulates granulocyte-macrophage colonystimulating factor production and inflammatory cell recruitment in the murine uterus. Biol Reprod. 1998; 58(5): 1217-1225.
- Askelund K, Liddell HS, Zanderigo AM, Fernando NS, Khong TY, Stone PR, et al. CD83(+) dendritic cells in the decidua of women

with recurrent miscarriage and normal pregnancy. Placenta. 2004; 25(2-3): 140-145.

- Spaggiari GM, Abdelrazik H, Becchetti F, Moretta L. MSCs inhibit monocyte-derived DC maturation and function by selectively interfering with the generation of immature DCs: central role of MSCderived prostaglandin E2. Blood. 2009; 113(26): 6576-6583.
- Harizi H, Gualde N. Pivotal role of PGE2 and IL-10 in the crossregulation of dendritic cell-derived inflammatory mediators. Cell Mol Immunol. 2006; 3(4): 271-277.
- Esebanmen GE, Langridge WHR. The role of TGF-beta signaling in dendritic cell tolerance. Immunol Res. 2017; 65(5): 987-994.
- Liu L, Zhao G, Fan H, Zhao X, Li P, Wang Z, et al. Mesenchymal stem cells ameliorate Th1-induced pre-eclampsia-like symptoms in mice via the suppression of TNF-a expression. PLoS One. 2014; 9(2): e88036.
- 40. Jing Z, Qiong Z, Yonggang W, Yanping L. Rat bone marrow mesenchymal stem cells improve regeneration of thin endometrium in rat. Fertil Steril. 2014; 101(2): 587-594.

Pluripotency Potential of Embryonic Stem Cell-Like Cells Derived from Mouse Testis

Hossein Azizi, Ph.D.1*, Behruz Asgari, M.Sc.², Thomas Skutella, Ph.D.³

1. Faculty of Biotechnology, Amol University of Special Modern Technologies, Amol, Iran 2. Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

3. Institute for Anatomy and Cell Biology, Medical Faculty, Heidelberg University, Im Neuenheimer Feld, Heidelberg, Germany

*Corresponding Address: P.O.Box: 46168-49767, Faculty of Biotechnology, Amol University of Special Modern Technologies, Amol, Iran Email: h.azizi@ausmt.ac.ir

Received: 5/June/2018, Accepted: 21/October/2018

Abstract

Objective: During the cultivation of spermatogonial stem cells (SSCs) and their conversion into embryonic stem-like (ES-like) cells, transitional ES-like colonies and epiblast-like cells were observable. In the present experimental study, we aimed to analyze the efficiency of the multipotency or pluripotency potential of ES-like cells, transitional colonies and epiblast-like cells.

Materials and Methods: In this experimental study, SSCs were isolated from transgenic octamer-binding transcription factor 4 (Oct4)-green fluorescent protein (GFP)-reporter mice. During cell culture ES-like, transitional and epiblast-like colonies developed spontaneously. The mRNA and protein expression of pluripotency markers were analyzed by Fluidigm real-time polymerase chain reaction (RT-PCR) and immunocytochemistry, respectively. Efficiency to produce chimera mice was evaluated after injection of ES and ES-like cells into blastocysts.

Results: Microscopic analyses demonstrated that the expression of Oct4-GFP in ES-like cells was very strong, in epiblast-like cells was not detectable, and was only partial in transitional colonies. Fluidigm RT-PCR showed a higher expression of the germ cell markers Stra-8 and *Gpr-125* in ES-like cells and the pluripotency genes *Dppa5*, *Lin28*, *Klf4*, Gdf3 and Tdgf1 in ES-like colonies and embryonic stem cells (ESCs) compared to the epiblast-like and transitional colonies. No significant expression of Oct-4, Nanog, Sox2 and c-Myc was observed in the different groups. We showed a high expression level of Nanog and Klf4 in ES-like, while only a partial expression was observed in transitional colonies. We generated chimeric mice after blastocystic injection from ES and ES-like cells, but not from transitional colonies. We observed that the efficiency to produce chimeric mice in ES cells was more efficient (59%) in comparison to ES-like cells (22%).

Conclusion: This new data provides more information on the pluripotency or multipotency potentials of testis-derived ES-like cells in comparison to transitional colonies and epiblast-like cells.

Keywords: Mouse Testis, Pluripotency Potential, Spermatogonial Stem Cells

Cell Journal(Yakhteh), Vol 21, No 3, October-December (Autumn) 2019, Pages: 281-289

Citation: Azizi H, Asgari B, Skutella T. Pluripotency potential of embryonic stem cell-like cells derived from mouse testis. Cell J. 2019; 21(3): 281-289. doi: 10.22074/cellj.2019.6068.

Introduction

It is well known that mouse spermatogonial stem cells (SSCs) are unipotent stem cells, which express both pluripotency and germ cell markers (1-3). SCCs are capable of spontaneously transforming into pluripotent embryonic stem (ES)-like cells under germ cell culture conditions without the artificial addition of exogenous pluripotency genes or small molecules (1, 4, 5). These pluripotent ES-like cells in turn can convert into several cell lineages including the three embryonic germ layers and germ cells (1, 5-8).

Kanatsu-Shinohara et al. (5), produced ES-like cells within 4-7 weeks post-culture initiation in neonatal SSC cultures for the first time, and were followed by other groups, who showed that these pluripotent stem cells can be derived from the murine testis cells from up to 7-week old adolescent mice (1, 5). In the study by Kanatsu-Shinohara et al. (5), the ES-like cells played an active part in their transformation into germline chimeras following injection into blastocysts. These findings demonstrated the pluripotency of SSCs or neonatal testis-derived gonocytes; however, the derivation processes of ES-like cells from SSCs remained unclear. Other more studies have successfully generated different populations of Stra8-positive, GPR125-positive and Oct4-positive SSCs from ES-like cells (4, 6, 7). In a study by Guan et al. (7)multipotent ES-like cells could be derived from Stra8positive SSCs from 7 week old mice, in vitro. Similar to the study by Kanatsu-Shinohara et al. (5) under in vitro and in vivo conditions, these cells could differentiate into all three germ layers in vitro and produced teratomas. After injection of Stra8-positive SSCs into blastocysts chimeras was formed (7). After mating, the chimera transmission to the next generation was observed. Germline transmission of Stra8-GFP-positive ES-like cells was not evaluated. Ko et al. (4) repeated the induction of pluripotency in 5-7 weeks Oct-4-GFP-positive adolescent SSCs. The authors described that the induction of differentiation dependends on the initial number of plated SSCs and the length of Oct4-positive cell culturing time without splitting. They manually picked the heterogonous Oct4-GFP-positive SSCs and demonstrated the relation between a certain number of SSCs (1000-4000) and a culture duration of 2-4 weeks for the induction of pluripotency. In a published protocol, this group described the conversion of SSCs into pluripotent stem cells only with SSCs of adolescent mice from postnatal day 35 (5 weeks old). The generated cells fulfilled the same criteria described by Kanatsu-Shinohara et al. (5) and Guan et al. (7). In another study this group generated ES-like cells from unselected testis cells of a testis biopsy (9). Seandel et al. (6) produced adult spermatogonial-derived stem cells from GPR-125 LacZ-positive cells in 3-week to 8-month old mice, but these cells were only multipotent, because no germline transmission was observed in the chimera.

Although we have not come to a complete understanding of the reprogramming mechanism and the establishment of ES-like cells from SSCs, it is obvious that the reprogramming process is influenced by various conditions. These include the age of the donor animals the SSC plating density, the time period post-culture initiation, the culture duration, and the cell population variations observed while in culture (1, 5, 7, 10). Furthermore, during conversion to pluripotent cells different types of colonies can be observed, including ES-like cells, epiblast-like cells and semi-transmitted transitional colonies (1, 6, 7).

In the current study, the multipotency or pluripotency potentials of testis-derived ES-like cells, epiblast-like cells and transitional colonies were examined by using molecular characterizations and chimera assays in comparison to ESCs.

Materials and Methods

Isolation of embryonic stem-like cells, epiblast-like cells and transitional colonies

All animal care was performed according to guidelines of the Institute for Anatomy and Cell Biology of Heidelberg University (Heidelberg, Germany) and the Royan Institutional Review Board and Institutional Ethical Committee (Tehran, Iran). Testis cells were isolated from 4-week old C57BL/6 Oct4-promoter reporter GFP transgenic mice after decapsulation and treatment by a one-step enzymatic digestion protocol. Germline stem cells (GSCs) were established according to our previous study (1). The above-mentioned produced colonies were sub-cultured in mouse ES cell medium with KnockOut[™] Dulbecco's Modified Eagle's Medium (KO-DMEM) or DMEM high-glucose medium (Invitrogen, USA), supplemented with 15% fetal bovine serum (FBS, Invitrogen, USA), 1% Non-Essential Amino Acid (NEAA) solution (Invitrogen, USA), 1% L-glutamine (Invitrogen, USA), 1% Pen-Strep (PAA) (Invitrogen, USA), 0 .1% β -mercaptoethanol (Invitrogen, USA) and leukemia inhibitory factor (LIF, Millipore, USA) at a final concentration of 1000 U/ml (1).

Fluidigm biomark system gene expression analyses

The expression of various pluripotency- and germ cellassociated genes *Oct4*, *Nanog*, *Sox2*, *Klf4*, *c-Myc*, *Lin28*, Gdf3, Tdgf1, Dppa-5, Stra8 and Gpr-125 was analyzed utilizing dynamic array chips (Table 1). The housekeeping gene, *Gapdh*, was selected for normalization of data in different cultured cell types, including ESCs, ES-like cells, epiblast-like cells and transitional colonies. The expression fold change of mRNA was compared to mouse embryonic fibroblasts (MEF) feeder cells as an additional control. With the help of a micromanipulator (Narashige Instruments) about 50 cells were manually selected from each sample. Afterwards, the selected cells were lysed with a special lysis buffer containing 9 µl RT-PreAmp Master Mix (5.0 µl Cells Direct 2× Reaction Mix) (Invitrogen, USA), 2.5 µl 0.2× assay pool, 0.2 µl RT/Taq Superscript III (Invitrogen, USA) and 1.3 µl TE buffer and directly frozen and stored at -80°C. The targeted transcripts were quantified with TaqMan real-time PCR on the BioMark real-time quantitative PCR (qPCR) system (Fluidigm, USA), with TaqMan gene expression assays (Invitrogen, USA) in 48.48 dynamic arrays. Two technical replicates were processed to analyze every sample. The CT values were analysed with GenEx software from MultiD, Excel and SPSS (1, 3, 11).

Immunocytofluorescent staining

For immunocytochemistry each cell type was cultured in 24-well plates and fixated in 4% paraformaldehyde. After rinsing, the samples were permeabilized with 0.1% Triton/phosphate buffered saline (PBS, Sigma, USA) and unspecific staining sites were blocked with 1% bovine serum albumin (BSA)/PBS. The cells were incubated overnight with primary antibodies for Nanog (Abcam, USA) and Klf4 (Cell Signaling, USA). After rinsing several times with PBS, the cells were incubated with species-specific secondary antibodies conjugated to different fluorochromes. Afterwards, the stained cells were counterstained with DAPI (0.2 μ g/ ml 4', 6-diamidino-2-phenylindole) (Sigma, USA) for 3 minutes at room temperature and fixed with Mowiol 4-88 reagent (Sigma, USA). As a negative control for all antibodies, the omission of each primary antibody in the sample was performed. The labeled cells were examined with a confocal microscope (Zeiss LSM 700) and images were obtained using a Zeiss LSM-TPMT (1, 2).

Production of chimeric mice

The differentiation potentials of ES cells and ESlike cells *in vivo* was examined utilizing chimera generation. At 3.5 days post-coitus, blastocysts were harvested from super-ovulated female mice and placed in M2 medium. Subsequently, 10-15 single-cell colonies were transferred into each blastocyst. About 10 injected embryos were surgically transplanted into the uterine horns of pseudo-pregnant recipient female mice. The coat color of the chimera mice was used for their identification (1).

Table 1: List of the TaqMan gene expression assays for multiplex quantitative reverse transcription-polymerase chain reaction (qRTPCR)

Gene	Gene name	Species	Assay ID
Dazl	Deleted in azoospermia-like	Mouse	Mm00515630_m1
Ddx4 or Vasa	DEAD (Asp-Glu-Ala-Asp) box polypeptide 4	Mouse	Mm00802445_m1
Zbtb16 or Plzf	Zinc finger and BTB domain containing 16	Mouse	Mm01176868_m1
Stra-8	Stimulated by retinoic acid gene 8	Mouse	Mm01165142_m1
Nanog	Nanog homeobox	Mouse	Mm02384862_g1
Lin28a	Lin-28 homolog A (C. elegans)	Mouse	Mm00524077_m1
Tdgfl	Teratocarcinoma-derived growth factor 1	Mouse	Mm00783944_g1
Dppa5	Developmental pluripotency associated 5	Mouse	Mm01171664_g1
Gdf3	Growth differentiation factor 3	Mouse	Mm00433563_m1
Pou5f1 or Oct-4	POU domain, class 5, transcription factor 1	Mouse	Mm03053917_g1
Sox2	SRY (sex determining region Y)-box 2	Mouse	Mm00488369_s1
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	Mouse	Mm999999915_g1

Statistical analysis

The experiments were repeated at least three times. The average gene expression in each group was quantified, and One-way analysis of variance (ANOVA) followed by the Tukey's post-hoc tests was employed to evaluate the experimental results.

Results

Characterization of embryonic stem-like cells, epiblast-like cells and transitional colonies

The characterization of the GSCs was established as described in our previous study (1). During passages of GSCs, we rarely found colonies which were similar to mouse ESCs that expressed high levels of Oct4-GFP, transitional colonies with partial expression of Oct4GFP, or and epiblast-like cells without expression of Oct4-GFP. About two months after initiation of GSC cultivation, according to morphological criteria and the re-occurring Oct4-GFP reporter signal, ESlike colonies, epiblast-like colonies and transitional colonies were observed (Fig.1).

The ES-like colonies had a packed spindle- to round-shaped morphology with smooth borders and expressed the Oct4-GFP signal at a very high intensity throughout the whole area of the colonies (Fig.1A). In contrast, the epiblast-like cell colonies had a flat morphology with no expression of Oct4-GFP (Fig.1B). The transitional cell colonies were characterized by a jagged, irregular or uneven border with a partial expression of Oct4-GFP in some areas of the colonies (Fig.1C).

Pluripotency Potential of ES-Like Cells from Mouse Testis



Fig.1: Different types of colonies are observed in spermatogonial stem cells (SSCs) cultures. Cell morphology and Oct4-GFP signals in A1. ESlike colonies, B1. Epiblast-like cells, C1. Transitional colonies, A2, B2, and C2. Show expression level of Oct4-GFP in the related cells (scale bar: 100 μ m).

In the next step, we examined the expression of pluripotency markers with Fluidigm RT-PCR for the ES cells, the ES-like, epiblast-like, and transitional colonies (Fig.2). The germ cell markers Stra8 and Gpr125 were expressed more strongly in the ESlike cells compared to any other group (P<0.05, Fig.2). Furthermore, higher expression levels of the pluripotency genes Klf4, Gdf3 and Lin28 were observed in ES-like cells and ESCs in comparison to the epiblast-like and transitional colonies (Fig.2). We also observed a significantly higher expression of *Tdgf1* in ESCs in comparison to the other groups (P<0.05, Fig.2). While the expression of *Dppa5* in the ES-like cells was significantly higher than in the other groups, we did not observe any significant difference in the expression of Oct4, Nanog, Sox2 and c-Myc among the groups (Fig.2).

Furthermore, by immunocytochemistry we detected Oct4-GFP positive cells in the ES-like colonies that were strongly stained for Nanog (Fig.3A1-3), and KLF4 (Fig.3, B1-3), while the transitional colonies only partially expressed Oct4-GFP and Nanog. Similar to the ES-like cells, in the transitional colonies there were areas that were partially positive for both Oct4-GFP and KLF4 (Fig.3).













Fig.2: mRNA expression of pluripotency and germ cell genes. Analysis was performed comparing embryonic stem cells (ESCs), ES-like colonies, epiblast-like cells (EP) and transitional colonies (TR). Y-axis denotes fold change of mRNA expression in comparison to MEF feeder cells. Significance of the difference between the different groups was determined with t test. a; At least P<0.05 versus other groups, b; At least P<0.05 versus EP and TR cells, and MEF; Mouse embryonic fibroblasts.

Chimeric mice production

In an additional experiment, we investigated the efficiency of the generation of chimeric mice with blastocyst injection of mouse ESCs and ES-like cells. We injected 164 embryos with ESCs and 169 embryos with ES-like cells that differentiated into blastocysts, which were implanted into a foster mother. We observed after embryo transfer 86 live births from ESCs and 41 from the ES-like group. The efficiency of the production of chimeric mice with ESCs was more efficient than the ES-like cells. Overall, 51 (59.3%) chimeric mice were generated after injection of ESCs and 9 (22%) chimeras from ES-like cells.



Fig.3: Immunocytochemical characterization of reprogrammed embryonic stem (ES)-like and transitional colonies. A1-A3. Oct4-GFP positive cells in the ES-like colonies are positive for the markers of Nanog, B1-B3. KIf4, while transitional colonies partially express Oct4-GFP signal, C1-C3. Nanog, and D1-D3. KLF4 (scale bar: 50 μm).

Discussion

In our study we demonstrated that ES-like, epiblast-like and transitional colonies all emerge during the conversion stage of unipotent GSCs into pluripotent cells, but only ES-like cells possess pluripotent stem cell potentials. Epiblast-like colonies are Oct4-GFP negative and are unable to shift to a pluripotent state. Transitional colonies are heterogeneous with only a partial expression of pluripotency genes. Only the strongly Oct4-GFP positive ES-like cells expressed a full network of pluripotency genes and contributed to the chimera assays. On the other hand, and in contrast to ESCs, the ES-like cells in parallel still strongly express the germ cell specific genes *Stra-8* and *Gpr-125*.

It is well documented that the transcriptional factor network of Oct-4, Nanog and Sox2 controls the pluripotency state in ESCs and is essential for the reprogramming of somatic cells into induced pluripotent stem cells (IPSCs) (12). This network blocks the expression of different lineage-specific genes and thus sustains pluripotency of the cells, preventing their differentiation (13). The level of expression of pluripotency gene is critical for the maintenance of pluripotency state (3, 14). In the colonies from the Oct4-GFP reporter mice, the GFP signal was strongly present only in the ES-like cells and partially in the transitional colonies. We confirmed that the expression of the pluripotency markers Nanog, and C-Myc in ES-like, transitional and epiblast-like cells was at similar levels in comparison to ESCs, while Sox2 was only strongly expressed in mouse ESCs. Our study also demonstrated partial and low expression levels of *Lin28* and *Klf4* in transitional and epiblast-like cells. The mRNA expression profiling confirmed that the expression levels of pluripotency markers were not the same, and significant differences were detected even between mouse ESCs and ES-like cells.

It has been suggested that the Oct4 protein, encoded by the Pou5f1 gene, is absolutely required for the stemness properties of ESCs. During early embryonic development, Oct4 is expressed in blastomeres and in the inner cell mass (ICM) of the blastocysts, from which ESCs are derived in vitro (15, 16). After gastrulation, the Oct4 protein is down-regulated in the trophectoderm and in the primitive endoderm, but is maintained in primordial germ cells (PGCs). PGCs continue to express of Oct4 until the initiation of spermatogenesis in males or oogenesis in females (16). In concert with Oct4 and Sox2, the transcription factor Nanog is a key factor to establish ESC identity and to maintain pluripotency. Nanog seems to maintain self-renewal in mouse ESCs with an independent mechanism of the leukemia inhibitor factor (LIF)/signal transducer and activator of transcription 3 (Stat3) signalling pathway. The deletion of Nanog in mouse ESCs leads to a loss of the pluripotency state and induces differentiation into the extra-embryonic endoderm cell lineage (17). It has been shown that SSCs express all of the different Yamanaka factors (Oct3/4, Sox2, Klf4, c-Myc) at the mRNA level (11, 18), while mRNA for Sox2 is not translated into a protein (19). It has been documented that blastocysts with a deficiency in Sox2 lose their pluripotent state, therefore are unable to shape a pluripotent ICM (20). Klf4 has also been shown to be an important transcription factor for the regulation of pluripotency in cells (21). Klf4 accompanied by the genes Oct4, Sox2 and c-Myc, is the main transcription factor for the generation of iPSCs from somatic cells (21, 22). Lin28 is a marker of ESCs and is expressed in undifferentiated mouse SSCs (23). Gillis et al. (24) reported that Lin28 might not be present in adult human testes, but they observed a high expression in human testicular germ cell tumors. We reported significantly high expression levels of Lin28 in mouse ES-like cells and ESCs compared to both epiblast-like and transitional cells.

There are diverse controversial challenges about the

pluripotency and multipotency of ES-like cells (25-28), including germ cell contribution and germ cell transmission.

Although it seems that ES-like cells from 4-week old Oct4-promoter reporter GFP transgenic mice have pluripotency potentials, in our experiments the efficiency for the production of chimeric mice from ESCs and ES-like cells was different. Our analysis showed that although chimeric mice could be generated in 22% of the cases after injection of ES-like cells into blastocysts, the efficiency to produce chimeric mice was lower than that after injection of ESCs (59%). Therefore, although ES-like cells express pluripotency markers and produce chimera, the degree of chimerism is not the same as the mouse ESCs.

In previous studies, Kanatsu-Shinohara et al. (5) microinjected ES-like cells from neonatal mice into blastocysts and observed chimerism in 36% (13 of 36) of the newborn animals as judged by EGFP-positive cells. According to their findings, donor cells were found in the central nervous system, liver, heart, lung, somites, intestine, and also in the germ cells in the testis of a 6-week old animal. Two offspring were obtained after performing microinsemination with EGFP-positive spermatids. By using the tetraploid complementation technique embryos, this group could generate embryos but no living offspring. This observation was explained by the altered imprinting status of the germ stem cells in comparison to mouse ES cells. Guan et al. (7) microinjected blastocysts with SSCs from 4 week old mice and detected chimaerism in 39 of 42 of mice (93%). After mating of chimeric males and females, these authors observed germline transmission. Furthermore, Ko et al. (4) performed chimera assays and observed germline transmission with mES-like cells at a lower level than with mouse ES cells.

Seandel et al. (6) generated GPR125 positive multipotent ES-like cells, which contributed to all three germ layers in embryoid body (EB) cultures and teratoma assays, but did not show germline transmission in chimeric embryos. Naive and prime pluripotency states have been demonstrated in pluripotent cells (29-32). Primary pluripotent stem cells, similar to late epiblast cells or postimplantation epiblast cells, could only produce chimeric animals to a limited extent (30). Therefore, it might be argued that the ES-like cells generated by Seandel et al. (6) were primary pluripotent cells (7).

To better understand the origin of the pluripotent stem cells, Guan et al. (33) generated ES-like cells from Stra8-GFP mice, while Seandel et al. (6) utilized GPR125 LacZ mice. However, the resulting cell lines were not from an initial germ cell population grown from a single cell (clonal growth) and from an ideal ES morphology (transitional morphology for Seandel) (4). Ko et al. (4) described clonal generation of ES-like cells, but the source of SSCs is unclear, because at a closer look Oct4-GFP SSCs lose the fluorescent signal after an initial germ cell culture (1, 4). Therefore, the ES-like cells, in which a strong GFP signal re-occurs, might have been generated from different types of SSCs or even other cells. In the past years, the enhancement of ES-like cell production by certain chemicals has been proven. The derivation of pluripotent ES-like cells could be expanded by glycogen synthase kinase-3 inhibition in neonate mouse testicular cultures (28).

Conclusion

During the culture of mouse testicular GSCs from 4-7 week old mice, different types of colonies are spontaneously generated, while only ES-like colonies are able to reach a full pluripotent state. The different types of colonies can be distinguished by morphological criteria. Only ES-like and transitional colonies show Oc4-GFP reporter signals, "epiblast like" colonies are Oct4-GFP negative. In contrast to ESCs, Oct4-GFP positive ES-like cells still express the germ cell specific genes Stra8 and GPR125, indicating that the Oct4-GFP positive ES-like cells maintaine their original epigenetic "germ cell memory". The remaining epigenetic germ cell-associated traces have to be further researched in the future. This observation might also be interesting for the generation of germ cells from pluripotent ES-like cells. The efficiency to produce chimeric mice was more efficient with mouse ESCs in comparison to ES-like cells. Further research of chimeric mouse production by ES-like cells has to be conducted in higher numbers, while analyzing the potency of these cells for germline transmission in more detail.

Acknowledgements

This study was financially supported by the Iranian National Science Foundation (INSF), the International Scientific Meeting Office, Ministry of Science, Research and Technology of Islamic Republic of Iran, and the University of Heidelberg, Institute for Anatomy and Cell Biology III, Department of Neuroanatomy, Germany. There is no conflict of interest in this study.

Authors' Contributions

H.A.; Wrote the manuscript, carried out and study design the experiment. B.A., T.S.; Participated in evaluation, critical feedback and data analysis. T.S.; Edited the manuscript. The authors read and approved the final manuscript.

References

- 1. Azizi H, Conrad S, Hinz U, Asgari B, Nanus D, Peterziel H, et al. Derivation of pluripotent cells from mouse SSCs seems to be age dependent. Stem Cells Int. 2016; 2016: 8216312.
- Azizi H, Skutella T, Shahverdi A. Generation of mouse spermatogonial stem-cell-colonies in a non-adherent culture. Cell J. 2017; 19(2): 238-249.
- Conrad S, Azizi H, Hatami M, Kubista M, Bonin M, Hennenlotter J, et al. Expression of genes related to germ cell lineage and pluripotency in single cells and colonies of human adult germ

stem cells. Stem Cells Int. 2016; 2016: 8582526.

- Ko K, Tapia N, Wu G, Kim JB, Bravo MJ, Sasse P, et al. Induction of pluripotency in adult unipotent germline stem cells. Cell Stem Cell. 2009; 5(1): 87-96.
- Kanatsu-Shinohara M, Inoue K, Lee J, Yoshimoto M, Ogonuki N, Miki H, et al. Generation of pluripotent stem cells from neonatal mouse testis. Cell. 2004; 119(7): 1001-1012.
- Seandel M, James D, Shmelkov SV, Falciatori I, Kim J, Chavala S, et al. Generation of functional multipotent adult stem cells from GPR125+ germline progenitors. Nature. 2007; 449(7160): 346-350.
- Guan K, Nayernia K, Maier LS, Wagner S, Dressel R, Lee JH, et al. Pluripotency of spermatogonial stem cells from adult mouse testis. Nature. 2006; 440(7088): 1199-1203.
- Bojnordi MN, Azizi H, Skutella T, Movahedin M, Pourabdolhossein F, Shojaei A, et al. Differentiation of spermatogonia stem cells into functional mature neurons characterized with differential gene expression. Mol Neurobiol. 2017; 54(7): 5676-5682.
- Ko K, Wu G, Arauzo-Bravo MJ, Kim J, Francine J, Greber B, et al. Autologous pluripotent stem cells generated from adult mouse testicular biopsy. Stem Cell Rev. 2012; 8(2): 435-444.
- Conrad S, Renninger M, Hennenlotter J, Wiesner T, Just L, Bonin M, et al. Generation of pluripotent stem cells from adult human testis. Nature. 2008; 456(7220): 344-349.
- Conrad S, Azizi H, Skutella T. Single-cell expression profiling and proteomics of primordial germ cells, spermatogonial stem cells, adult germ stem cells, and oocytes. Adv Exp Med Biol. 2018 (ahead of print).
- Cai Y, Dai X, Zhang Q, Dai Z. Gene expression of OCT4, SOX2, KLF4 and MYC (OSKM) induced pluripotent stem cells: identification for potential mechanisms. Diagn Pathol. 2015; 10: 35.
- Boyer LA, Lee TI, Cole MF, Johnstone SE, Levine SS, Zucker JP, et al. Core transcriptional regulatory circuitry in human embryonic stem cells. Cell. 2005; 122(6): 947-956.
- Taleahmad S, Mirzaei M, Parker LM, Hassani SN, Mollamohammadi S, Sharifi-Zarchi A, et al. Proteome analysis of ground state pluripotency. Sci Rep. 2015; 5: 17985.
- Schöler HR. Octamania: the POU factors in murine development. Trends Genet. 1991; 7(10): 323-329.
- 16. Pesce M, Schöler HR. Oct-4: control of totipotency and germline determination. Mol Reprod Dev. 2000; 55(4): 452-457.
- Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, et al. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell. 2003; 113(5): 631-642.
- Conrad S, Azizi H, Hatami M, Kubista M, Bonin M, Hennenlotter J, et al. Differential gene expression profiling of enriched human spermatogonia after short- and long-term culture. Biomed Res Int. 2014; 2014: 138350.
- Imamura M, Miura K, Iwabuchi K, Ichisaka T, Nakagawa M, Lee J, et al. Transcriptional repression and DNA hypermethylation of a small set of ES cell marker genes in male germline stem cells. BMC Dev Biol. 2006; 6: 34.
- Masui S, Nakatake Y, Toyooka Y, Shimosato D, Yagi R, Takahashi K, et al. Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells. Nat Cell Biol. 2007; 9(6): 625-635.
- 21. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006; 126(4): 663-676.
- Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007; 131(5): 861-872.
- Zheng K, Wu X, Kaestner KH, Wang PJ. The pluripotency factor LIN28 marks undifferentiated spermatogonia in mouse. BMC Dev Biol. 2009; 9: 38.
- Gillis AJ, Stoop H, Biermann K, van Gurp RJ, Swartzman E, Cribbes S, et al. Expression and interdependencies of pluripotency factors LIN28, OCT3/4, NANOG and SOX2 in human testicular germ cells and tumours of the testis. Int J Androl. 2011; 34(4 Pt 2): e160-174.
- de Rooij DG, Mizrak SC. Deriving multipotent stem cells from mouse spermatogonial stem cells: a new tool for developmental and clinical research. Development. 2008; 135(13): 2207-2213.
- 26. Ko K, Araúzo-Bravo MJ, Tapia N, Kim J, Lin Q, Bernemann

C, et al. Human adult germline stem cells in question. Nature. 2010; 465(7301): E1; discussion E3.

- 27. Tapia N, Araúzo-Bravo MJ, Ko K, Schöler HR. Concise review: challenging the pluripotency of human testis-derived ESC-like cells. Stem Cells. 2011; 29(8): 1165-1169.
- Moraveji SF, Attari F, Shahverdi A, Sepehri H, Farrokhi A, Hassani SN, et al. Inhibition of glycogen synthase kinase-3 promotes efficient derivation of pluripotent stem cells from neonatal mouse testis. Hum Reprod. 2012; 27(8): 2312-2324.
- 29. Nichols J, Smith A. Pluripotency in the embryo and in culture. Cold Spring Harb Perspect Biol. 2012; 4(8): a008128.
- 30. Nichols J, Smith A. Naive and primed pluripotent states. Cell

Stem Cell. 2009; 4(6): 487-492.

- Hassani SN, Totonchi M, Gourabi H, Schöler HR, Baharvand H. Signaling roadmap modulating naive and primed pluripotency. Stem Cells Dev. 2014; 23(3): 193-208.
- 32. Hassani SN, Pakzad M, Asgari B, Taei A, Baharvand H. Suppression of transforming growth factor β signaling promotes ground state pluripotency from single blastomeres. Hum Reprod. 2014; 29(8): 1739-1748.
- Guan K, Wolf F, Becker A, Engel W, Nayernia K, Hasenfuss G. Isolation and cultivation of stem cells from adult mouse testes. Nat Protoc. 2009; 4(2): 143-154.

Gene Expression Patterns of Royan Human Embryonic Stem Cells Correlate with Their Propensity and Culture Systems

Hassan Rassouli, M.Sc.^{1#}, Mona Khalaj, M.Sc.^{1#}, Seyedeh-Nafiseh Hassani, Ph.D.², Shiva Nemati, Ph.D.², Ghasem Hosseini Salekdeh, Ph.D.^{1, 3, 4*}, Hossein Baharvand, Ph.D.^{2, 5*}

1. Department of Molecular Systems Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

2. Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

3. Department of Systems Biology, Agricultural Biotechnology Research Institute of Iran, Agricultural Research, Education, and Extension Organization, Karaj, Iran

4. Department of Molecular Sciences, Macquarie University, Sydney, NSW, Australia

5. Department of Developmental Biology, University of Science and Culture, Tehran, Iran

#The first two authors equally contributed to this work.

*Corresponding Addresses: P.O.Box: 16635-148, Department of Molecular Systems Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

P.O.Box: 16635-148, Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

Emails: salekdeh@royanInstitute.org, baharvand@royaninstitute.org

Received: 30/June/2018, Accepted: 20/November/2018 Abstract

Objective: Human embryonic stem cells (hESCs) have the potential to give rise to all types of cells in the human body when appropriately induced to differentiate. Stem cells can differentiate spontaneously into the three-germ layer derivatives by embryoid bodies (EBs) formation. However, the two-dimensional (2D) adherent culture of hESCs under defined conditions is commonly used for directed differentiation toward a specific type of mature cells. In this study, we aimed to determine the propensity of the Royan hESC lines based on comparison of expression levels of 46 lineage specific markers.

Materials and Methods: In this experimental study, we have compared the expression of lineage-specific markers in hESC lines during EB versus adherent-based spontaneous differentiation. We used quantitative real-time polymerase chain reaction (qRT-PCR) to assess expressions of 46 lineage-specific markers in 4 hESC lines, Royan H1 (RH1), RH2, RH5, and RH6, during spontaneous differentiation in both EB and adherent cultures at 0, 10, and 30 days after initiation of differentiation.

Results: Based on qRT-PCR data analysis, the liver and neuronal markers had higher expression levels in EBs, whereas skin-specific markers expressed at higher levels in the adherent culture. The results showed differential expression patterns of some lineage-specific markers in EBs compared with the adherent cultures.

Conclusion: According to these results, possibly the spontaneous differentiation technique could be a useful method for optimization of culture conditions to differentiate stem cells into specific cell types such ectoderm, neuron, endoderm and hepatocyte. This approach might prove beneficial for further work on maximizing the efficiency of directed differentiation and development of novel differentiation protocols.

Keywords: Differentiation, Gene Expression, Pluripotency, Propensity, Stem Cell

Cell Journal(Yakhteh), Vol 21, No 3, October-December (Autumn) 2019, Pages: 290-299 _

Citation: Rassouli H, Khalaj M, Hassani SN, Nemati Sh, Hosseini Salekdeh Gh, Baharvand H. Gene expression patterns of Royan human embryonic stem cells correlate with their propensity and culture systems. Cell J. 2019; 21(3): 290-299. doi: 10.22074/cellj.2019.6128.

Introduction

Embryonic stem cells (ESCs) divide indefinitely and give rise to all different cell types during differentiation. These unique properties make them invaluable cell sources for a wide range of applications in regenerative medicine, cell therapy, disease modeling, drug screening, gene delivery, and other research. However, despite their potential, human ESCs (hESCs) have not been successfully introduced into the clinic setting. Numerous obstacles must be overcome prior to their efficient use in cell replacement therapies. In addition to ethical issues that concern the embryonic source of hESCs (1), the most important technical challenges that stem cell researchers face are concerns about the safety of the cells (2), their purity (3), host immune system rejection (4), efficiency and reproducibility of differentiation, and cell transduction or post-transplantation issues (5).

Optimization of the current differentiation protocols is a major challenge in stem cell research. The ability to efficiently generate pure populations of mature cell types from stem cells over a reasonable period of time is a major challenge. There are several protocols that enable differentiation of hESCs into diverse cell types like cardiomyocytes (6, 7), dopaminergic neurons (8), definitive endoderm (9), human hepatic competent endoderm (10), neural cells (11), and insulin-producing cells (12). Different approaches vary in terms of chemical and mechanical factors, and include the composition of growth factors and soluble inducers, forces applied in the cultured cells, and the culture system used for differentiation. Modifications in the chemical factors in stem cell media have been intensely analyzed in order to enhance differentiation. However, researchers have recently noted the significant role of physical and mechanical variables on differentiation efficiency (13). Cell-cell and cell-matrix interactions have significant roles in cell fate decisions (14). The combination of chemical and mechanical factors that regulate the stem cell fate *in vivo* can influence the direction and efficiency of lineage specifications *in vitro*. Therefore, depending on the mature cell type of interest, it is critical to provide stem cells with the appropriate culture system.

The following 3 main methods are commonly used for stem cell culture: i. Suspension culture and formation of embryoid bodies (EB), ii. Monolayer culture of stem cells on extracellular matrix proteins, and iii. Culture of ESCs on a feeder layer (15). Some protocols for directed differentiation of ESCs use an adherent culture system (16, 17), whereas others suggest a suspension culture (18-20). Advantages and disadvantages associated with both methods make it difficult to choose one method over the other. Stem cells are cultured in monolayers in the adherent system, and the cells divide and grow in a two-dimensional (2D) manner. The soluble inducing factors should be added to the medium for initiation of differentiation, which would allow the cells to have homogenous access to soluble factors in the medium. However, the 2D culture systems do not provide *in vivo* interactions like cell-cell and cell-matrix connections. The differentiation procedure in the suspension method is basically different compared to the adherent system. In the suspension culture, ESCs usually form threedimensional (3D) cellular aggregates that lead to the formation of EBs, which begin to spontaneously differentiate before the addition of any soluble inducers (21). EBs resemble the initial stages of embryonic development, in which they form primary embryonic germ layers (ectoderm, mesoderm, and endoderm) (22). The spatial interactions between cells in EBs help direct cell fate determination through signaling pathways that are mainly absent in adherent cultures (23-25). However, as these EBs increase in size, it is difficult for soluble factors to diffuse into the inner cell layers of the aggregate.

Some of the directed differentiation protocols include an initial step of EB mediated spontaneous differentiation followed by addition of soluble inducing factors to the cell aggregates. The differences in dimensions of the suspension and adherent culture systems result in significant differences between cellcell and cell-matrix interactions, which highlights the importance of a proper choice of a 2D versus 3D stem cell culture system. Several reports have theoretically compared the adherent and suspension culture systems and their effects on the overall differentiation efficiency of stem cells, but there is no report of an experimental comparison (26, 27). In theory, pros and cons exist for both culture systems, and the choice of the appropriate culture system necessitates experimental tests for detailed examination and comprehensive analysis.

Different signal transduction pathways play roles in the in vivo differentiation of stem cells into each type of mature cells. The microenvironmental factors that govern a cell's fate in the body include structural, biochemical, mechanical, physiological, and hydrostatic stimuli (28). The environmental triggers required to induce differentiation toward one lineage may differ completely from other lineages; thus, it is reasonable that the choice of culture system for directed differentiation must be cell type-specific. Some cell types might differentiate more efficiently under 2D conditions, whereas other cell types prefer 3D culture systems. Stem cell researchers are seeking straightforward and accurate experimental methods to provide critical information to maximize the efficiency of differentiation for specific cell types.

EB formation and spontaneous differentiation of ESCs result in heterogeneous populations of differentiated cells that include cells of the primary germ layers as well as some of the mature cell types that they produce. Spontaneous differentiation could be the first step for production of pure populations of desired cell types. The convenience of spontaneously differentiated ESCs makes them a useful research tool to provide researchers with valuable information about stem cell behavior, even during directed differentiation. It has been shown that comparative expression of lineagespecific markers during spontaneous differentiation of ESC lines is a good representative of their relative potentials for directed differentiation toward different lineages (29, 30). In these studies, researchers have analyzed the expression levels of lineage-specific markers at various time points after spontaneous differentiation of several distinct ESC lines. Based on the comparative marker expression levels of each lineage, the researchers could hypothesize which ESC line had a higher intrinsic propensity to differentiate toward a specific lineage or cell type. Directed differentiation was then used to validate their hypothesis. The results have proven that spontaneous differentiation is an informative method. Simplicity and reliable data over a relatively short period of time, as well as the cost effectiveness of spontaneous differentiation, make it an interesting approach to study stem cell behavior during differentiation. Since the neural cells differentiation potencies have great potential for cell therapy and treatment of neurological disorders (31).

In this study, we used spontaneous differentiation to optimize differentiation in 4 Royan hESC lines. These hESC lines were cultured under both adherent and suspension culture conditions and we compared expression levels of 46 lineage specific markers to determine the propensity of each of the Royan hESC lines. Subsequently, direct differentiation for neural cells and hepatocytes was done to confirm the spontaneous differentiation results.

We evaluated our hypothesis by focusing on the expression level of neural stem cells lineage specific markers (in two undifferentiated and differentiated states with adherent or suspension culture system) like NESTIN, SOX1, NEUROD1, NCAM, PAX6, PDGFRa and GFAP (as general neural stem cell markers), and b-Tubulin (neural differentiation marker) (32) in spontaneously differentiated samples and specific neural subtypes markers like TH (marker for dopaminergic neural subtypes) in samples from direct differentiation (33).

In the other side we evaluated hepatocyte differentiation potency by focusing on spontaneous and direct endodermal layer differentiation and checking the general hepatic lineage specific expression markers like BRACHYURY, GOOSECOID, and SOX17 in two different culturing strategies (adherent and suspension).

Materials and Methods

Human embryonic stem cell culture

In this experimental study, approved by the Ethical Committee of Royan Institute, we have used 4 hESC lines-Royan H1 (RH1), RH2, RH5, and RH6 (34). RH6 is a male hESC line, whereas the other 3 hESC lines have been derived from female embryos. These hESC lines all have a normal karyotype (35). Passage 15-20 hESCs were transferred to dishes coated with mouse embryonic fibroblasts (MEF) and we derived 3 different biological replicates from each cell line. The hESCs were cultured in standard Dulbecco's modified Eagle's media (DMEM, Gibco, USA) complemented 20% knockout serum replacement (KOSR), 2 mM L-glutamine, 0.1 mM β -mercaptoethanol (BME, Sigma, USA), 100 µg/ml pen/ strep, and 100 ng/ml basic fibroblast growth factor (bFGF, Royan Biotech, Iran) (36).

Spontaneous differentiation

In order to form EBs, we cultured the hESCs in suspension stem cell medium that contained agarose and fetal bovine serum (FBS, Gibco, USA), without KOSR. The samples were harvested at days 10 and 30 after the initiation of EB formation. For spontaneous differentiation in the adherent settings, we used the same media with slight changes. The cells were cultured on 0.1% gelatin coated plates instead of agarose coated plates.

RNA extraction and cDNA synthesis

Total RNA was extracted using TRIzol (Sigma, USA) according to the manufacturer's protocol. The RNA

concentration was measured using a Biowave WPA (S 2100) spectrophotometer. We examined the purity of each RNA sample based on 260/280 absorbance ratios. We noted that all samples had a ratio of 1.9-2. The quality of total RNA from all samples was determined by electrophoresis. Ribosomal 50S and 28S bands were sharp and showed approximately 2:1 band intensity, which confirmed that the samples had acceptable RNA integrity and quality.

We used a DNase treatment kit (Fermentas, USA) based on the manufacturer's instructions for elimination of DNA contamination. Random hexamer primers were used for first strand cDNA synthesis using the Fermentas kit.

Quantitative real-time polymerase chain reaction

Totally, we used 48 primers in this study. The sequences of 34 primers were obtained from (29) and 13 primers were designed using Gene Runner and PerlPrimer software (Table S1) (See Supplementary Online Information at www.celljournal.org). Primers were synthesized by Metabion Company. The quantitative real-time polymerase chain reaction (qRT-PCR) reactions were performed using a Corbett machine, 72-well rotor using SYBR Green from ABI (Applied Biosystems, USA). All qRT-PCR reactions were run in duplicate, and we used the average threshold cycle (Ct) of 2 duplicates for further analysis. We used the housekeeping genes, *GAPDH* and β -Actin, as they have the most homogenous expression level among the Royan hESC lines based on the NormFinder software analysis (data not shown). The geometric mean Ct of *GAPDH* and β -Actin were calculated for each sample, and the Ct results from all 46 genes were normalized based on the mean housekeeping values.

Directed neural differentiation of human embryonic stem cells

RH5 hESCs were differentiated to neuronal cells using 2 different protocols for confirmation of the spontaneous differentiation results. These protocols consisted of 3 main steps: i. Induction of hESC colonies toward neural ectoderm, ii. Differentiation toward neural tube formation, and iii. Neuron maturation stage (Fig.1A). Steps i and ii differed between the 2 protocols. In the first protocol, the cells were cultured in a suspension culture and they were grown in an adherent condition in the second protocol. The third step was identical for both protocols. Neural ectoderms were obtained by culturing hESCs in induction medium for 6 days, followed by 6 days in the same medium without Noggin. For induction of neural tube formation, the concentration of bFGF was increased to 25% and the cells were maintained in this medium for 6 days. For maturation, neural tubes (in adherent culture) and neuronal precursor cells (in suspension condition) were transferred to laminin/ poly-L-ornithine culture dishes and grown for 12-14

days in maturation medium. Samples were collected from all the 3 stages for both differentiation protocols.

Directed hepatic differentiation of human embryonic stem cells

We used 2 hESC lines (RH2, RH6) at passages 25-35 to differentiate into a hepatic lineage according to the protocol of Basma et al. (37) with some modifications (Fig.1B). Briefly, EBs were generated by plating collagenase/dispase-passaged cells at a density of 1-5×10⁴ cells/cm² on bacterial petri dishes for 48 hours in DMEM/F12 supplemented with 20% KOSR, 1 mM nonessential amino acids, and 2 mM L-glutamine. Then, EBs were plated on Matrigel-coated plates in DMEM/F12 supplemented with Activin A (100 ng/ ml) for 6 days to induce definitive endoderm lineage. The day-6 cells were used as definitive endoderm for analysis. The concentration of KOSR was 0% for the first 48 hours, 0.2% for the second 24 hours, and 2.0% for the final 24 hours. Cells were then grown for 3 days in DMEM/F12 that contained 2.0% KOSR, 1 mM nonessential amino acids, 2 mM L-glutamine, 1% dimethyl sulfoxide, 10 ng/ml fibroblast growth factor 4 (FGF4, Royan Biotech, Iran), and 20 ng/ ml bone morphogenetic protein 2 (BMP2, Sigma, USA). The cells were allowed to grow in the same base media for an additional 4 days with 100 ng/mL hepatocyte growth factor (HGF, Sigma, USA) instead of FGF4 and BMP2. Next, they were cultured for 7-8 additional days in hepatocyte culture medium (HCM, Lonza, Swiss) that contained 2% KOSR, 1 mM nonessential amino acids, 2 mM L-glutamine, and 50 ng/ml HGF for the first 2 days as pre-hepatocyte cells at this step, followed by 5-6 days in maturation media that contained the same base media with 20 ng/ ml oncostatin M (OSM, Royan Biotech, Iran), 10 ng/ ml HGF, and 0.1 µM dexamethasone.

Statistical analysis

qRT-PCR results were converted to relative concentrations based on the standard curve method. Analysis of variances was performed on the readings from 60 samples and 48 different transcripts. We used the Statistical Analysis System (SAS) for 2-factor ANOVA by considering the hESC lines and the culture methods as 2 variable factors. P<0.01 were considered to be significant. SAS also provides a Duncan grouping chart for each gene in which samples are sorted based on that gene's expression level. We used Eisen Lab and TreeView softwares for hierarchical clustering of the samples and genes.

Results

Gene expression profiles of the different hESC lines begin to diverge during differentiation

Clustering of the complete data set, as shown in Figure 1C, indicates that different hESC lines have similar

expression profiles at the undifferentiated stage. Once the cells start to spontaneously differentiate, their gene expression profiles differ significantly due to their distinct intrinsic lineage propensities. As expected, most of the genes that were markers of the same cell type were clustered in close proximity in the clustering tree, which indicated reliability of the results. Pluripotency markers Oct4, Nanog, and TDGF were all clustered together, as were the endoderm markers HNF3b, CXCR4, and SOX17.

CHD1 temporal expression pattern differed in comparison with pluripotency markers

CHD1, a chromatin remodeler known to be involved in formation and maintenance of the open chromatin state, showed interesting results. We assessed the expression levels of CHD1 in 4 hESC samples, and at days 10 and 30 after spontaneous differentiation. Figure 2A shows that the expression level of CHD1 increased upon differentiation and peaked at day 10, which was unexpected if it had a similar role in mouse ESCs and hESCs. A study on mouse ESCs showed that this protein highly expressed in stem cells and was responsible for the existence of a completely open chromatin in undifferentiated stem cells, and was required for the stemness property of mESCs.

Expression levels of lineage-specific markers showed variations among different cell lines

A comparison of lineage-specificity among the 4 hESC lines in the current study was the first step for additional approval of the reliability of marker expression levels in spontaneous differentiation. The majority of the 46 examined markers showed significant differences in gene expression patterns among the 4 lines, which suggested divergent lineage specification. Based on spontaneous differentiation results, we selected 2 hESC lines for directed differentiation tests. The RH2 line showed the highest propensity to express endodermal (Fig.2B) and hepatocyte markers (Fig.3A). RH6 showed the lowest propensity to express endodermal (Fig.2B) and hepatocyte markers (Fig.3A). RH5 had the highest relative propensity to express the mesodermal (Fig.3B) and neural markers (Fig.4). We selected the RH2 and RH6 lines for further study on the comparison of directed differentiation toward hepatocytes under identical conditions. qRT-PCR results of directed differentiation towards endodermal and hepatocytes showed that RH2 had significantly higher efficiency to differentiate to endoderm and hepatocytes (Fig.5). There was a substantial distance between RH2 and RH6 based on the hierarchical clustering tree for hepatic markers (Fig.S1) (See Supplementary Online Information at www.celljournal.org). These results confirmed previous researches on determination of lineage specificity among different stem cell lines (29, 30).



В



Fig.1: Differentiation protocols and clustering tree. **A.** The 3 steps of the neural induction and differentiation protocol, **B.** The 3 steps of the hepatic differentiation protocol, and **C.** Cluster of all samples based on expression levels of the 46 marker genes (vertical axis). hESCs; Human embryonic stem cells, N2; N2 supplement, RA; Retinoic acid, bFGF; Basic fibroblast growth factor, IM; Induction medium, AA; Amino acids, EB; Embryoid bodies, DMSO; Dimethyl sulfoxide, BMP; Bone morphogenetic protein, HGF; Hepatocyte growth factor, HCM; Hepatocyte culture medium, OSM; Oncostatin M, and Dex; Dexamethasone.


Fig.2: Expression comparison of spontaneous differentiation in different conditions. **A.** The expression level of CHD1 peaked at day 10 of spontaneous differentiation in both the suspension and adherent culture systems and **B.** Comparison of the expression levels of liver-specific markers between the Royan H2 (RH2) and RH6 lines during spontaneous differentiation in suspension [embryoid body (EB)] and adherent (AD) conditions.



Fig.3: Expression comparison of endodermal and mesodermal markers between Royan embryonic stem cell (ESC) lines. A. Comparison of the expression levels of endodermal markers between the Royan H2 (RH2) and RH6 cell lines during spontaneous differentiation in suspension [embryoid body (EB)] and adherent (AD) conditions and **B.** Comparison of the expression levels of mesodermal markers between RH6 and RH5 cell lines during spontaneous differentiation in EB and AD conditions.



Fig.4: Relative expression levels of 3 neural markers in the 4 human embryonic stem cell (hESC) lines during spontaneous differentiation in suspension [embryoid body (EB)] or adherent (AD) culture systems.

295

Different cell lines showed differentiation potential in suspension and adherent cultures

Primary results confirmed the effectiveness of spontaneous differentiation in a comparison of the different hESC lines. In the second step, we compared EB versus the adherent culture methods using the same approach. Gene expression levels in suspension and adherent culture were compared for all lines. Interestingly, different lineage markers showed differential patterns in each condition. The ectodermal, neural, endodermal, hepatic, and endothelial markers had higher expression levels in suspension condition (Fig.6A), while the expression of skin-specific markers was higher in the adherent system (Fig.6B).



Fig.5: Relative expression levels comparison between RH2 and RH6 cell lines. A. four endodermal markers and B. four hepatocyte markers in directed differentiation of the Royan H2 (RH2) and RH6 cell lines in 3 stages: endoderm (ENDO), pre-hepatocyte (PREHEP), and mature hepatocyte.



Fig.6: Expression comparison of neural and keratinocyte markers in adherent or suspension culture conditions. A. Relative expression levels of 6 neural markers in Royan H5 (RH5) during spontaneous differentiation under adherent or suspension culture conditions and B. Relative expression levels of keratinocyte markers, P63 and KRT14, under suspension [embryoid body (EB)] and adherent (AD) culture conditions in 4 human embryonic stem cells (hESC) lines (note the scale on the Y-axis).

Directed neural differentiation of human embryonic stem cells

We confirmed the spontaneous differentiation results by direct differentiation of the RH5 cell line into neural cells according to the 2 induction protocols. The first protocol comprised the suspension culture stage, whereas the second protocol used the adherent culture during differentiation. Results of directed differentiation of stem cells to neurons confirmed spontaneous differentiation results, which showed that the suspension protocol was significantly more effective than the adherent culture.

Our results confirmed that the expression level of neural specific expressing markers like GFAP, PAX6, PDGFRa (as general neural stem cell markers) and b-Tublin (as general pre-mature neuronal marker) are higher in suspension (EB form) than adherent system in both direct and spontaneous differentiation systems, even in neuro-ectodermal specification or neural maturation stages. Although in both differentiation systems we have acceptable increase in neural lineage specific markers (Fig.S2) (See Supplementary Online Information at www. celljournal.org).

Directed endodermal and hepatic differentiation of human embryonic stem cells

Spontaneous differentiation results showed that RH2 and RH6 had significant differences in expression of endoderm and hepatocyte markers. According to these results, RH2 differentiated into endodermal and hepatocyte cells with higher efficacy compared to RH6. Direct endodermal and hepatic differentiation confirmed that spontaneous differentiation could be a powerful tool to predict the propensity of hESC lines. Direct differentiation results verified findings that the RH2 cell line had significantly higher expression levels of endodermal and hepatic markers (Fig.5). According to these data, spontaneous differentiation analysis could be a reliable, rapid, and economical method for optimization of differentiation protocols.

Discussion

hESCs have remarkable potential as cell sources for cell-based therapies. However, lack of knowledge in many aspects of hESC biology is a main barrier for introduction of stem cells in the clinic. In this study, we have focused on optimization of hESCs differentiation toward desired lineages, as one of the most important challenges in stem cell applications. Prior to using stem cells in the clinic, 2 major obstacles must be solved-differentiation efficiency and purity of differentiated cells. However, both must have the capability to produce purified cells at the maximum rate. Optimization of current direct differentiation protocols can overcome these problems. Here, we have investigated the effects of 2 parameters, cell line and dimension (2D vs. 3D). Both parameters had a significant influence on the final results. Although the effect of dimension has been extensively discussed, there

are few reports that have compared different lineages in 2D versus 3D culture systems. More recently, the 3D culture systems have been developed to recapitulate human complicated organs (like nervous system) development and differentiation in in vitro system starting from human pluripotent stem cells (hPSCs) by organoid technology (cerebral organoid models). Although many studies have been conducted to reveal the mechanisms of 3D differentiation in higher organs studies but there are still many questions to be addressed (38). In the most suspension differentiation system, increases in expression levels of region specific neural genes are shown. That these changes in the expression level of specific genes are mostly described by mysterious cell-cell interaction and releasing neurotrophic factors from specific regions of EBs (spheroids), and 3D self-organization of ESCs in suspension culture system (39).

Needed cell types dictate using suspension or adherent culture systems

We observed that the ectodermal, neural, endodermal, hepatic, and endothelial markers had higher expression levels in cells grown in the suspension condition, while skin-specific markers were expressed more in the adherent system. A possible explanation could be the similarity of the ex vivo environment to the natural extracellular environment for each cell or tissue type (40). Keratinocytes usually grow in a 2D layered condition in the body; hence these cells would prefer the adherent culture system. On the other hand, neural cells and hepatocytes grow in the 3D state in organs. Thus, they had more efficient differentiation in suspension culture. Cell-cell interactions and some signaling pathways might also be involved in the hESC response to the culture condition. Further studies would be needed to reveal the mechanisms that underlie lineage fate determination of hESCs during early differentiation stages of different culture methods. According to the current study results, some of the differentiation protocols could be improved by addition of a suspension step in the early stages of these protocols.

Spontaneous differentiation has the potential to predict the behavior of hESC lines in direct differentiation

Gene expression profile analysis of hESCs during differentiation is a simple and reliable approach to predict their lineage propensities. This cost effective method could provide very useful data over a short period of time instead of cultivation of different hESC lines, differentiating them to all possible lineages, and comparing them. In the current study, we have used this invaluable tool to compare the expression levels of lineage-specific markers during spontaneous differentiation of hESC lines in suspension versus adherent culture systems. Although reports have shown this tool's usefulness, we decided to confirm spontaneous differentiation results by direct differentiation of the RH5 cell line to neural cells. The direct differentiation data verified previous results and proved that this tool could be useful for optimization of hESC differentiation. According to the current results, the addition of a suspension culture step in differentiation of most lineages would be necessary. This new strategy may lead to the optimization of some common protocols used for neural differentiation of stem cells, and provide a standard platform for analysis of other types of differentiation protocols.

Conclusion

Future studies, such as high-throughput analysis of the expression profiles on ESC lines during differentiation in adherent versus suspension culture conditions, are required for additional information in this area. Elucidation of the mechanisms that cause the early events of lineage specification under the 2 culture conditions is necessary. Commonly used differentiation protocols can be compared with respect to different environmental variables such as chemical and mechanical properties of the culture system to enhance the efficiency of stem cell differentiation toward a desired cell type, and further pave the way for stem cells to be used in a clinical setting.

Acknowledgements

This work was supported by a grant from Royan Institute and the Iran National Science Foundation (INSF) to H.B (89000045). The authors declare no financial and conflicts of interest.

Authors' Contributions

H.R., M.K.; Contributed to all experimental work, data and statistical analysis, manuscript writing and figures preparation. S.N.; Performed cell culture. S.-N.H.; Provided scientific advice throughout the project and performed cell culture. G.H.S., H.B.; Scientific supervisor of the project, main idea and finalized manuscript. All authors read and approved the final version of this manuscript.

References

- Volarevic V, Markovic BS, Gazdic M, Volarevic A, Jovicic N, Arsenijevic N, et al. Ethical and safety issues of stem cell-based therapy. Int J Med Sci. 2018; 15(1): 36-45.
- Shroff G, Barthakur JK. Safety of human embryonic stem cells in patients with terminal/incurable conditions-a retrospective analysis. Ann Neurosci. 2015; 22(3): 132-138.
- Bukys MA, Bakos BB, Áfelik Ś, Zimmerman B, Barbaro B, Lin DL, et al. Xeno-transplantation of macro-encapsulated islets and pluripotent stem cell-derived pancreatic progenitors without immunosuppression. J Stem Trans Bio. 2016; 2(1): 109.
- He J, Rong Z, Fu X, Xu Y. A safety checkpoint to eliminate cancer risk of the immune evasive cells derived from human embryonic stem cells. Stem cells. 2017; 35(5): 1154-1161.
- Schwartz SD, Regillo CD, Lam BL, Eliott D, Rosenfeld PJ, Gregori NZ, et al. Human embryonic stem cell-derived retinal pigment epithelium in patients with age-related macular degeneration and Stargardt's macular dystrophy: follow-up of two open-label phase 1/2 studies. Lancet. 2015; 385(9967): 509-516.
- Fonoudi H, Yeganeh M, Fattahi F, Ghazizadeh Z, Rassouli H, Alikhani M, et al. ISL1 protein transduction promotes cardiomyocyte differentiation from human embryonic stem cells. PLoS One. 2013; 8(1): e55577.
- 7. Ghazizadeh Z, Fattahi F, Mirzaei M, Bayersaikhan D, Lee J, Chae

S, et al. Prospective Isolation of ISL1+ Cardiac Progenitors from Human ESCs for Myocardial Infarction Therapy. Stem Cell Reports. 2018; 10(3): 848-859.

- Fathi A, Rasouli H, Yeganeh M, Salekdeh GH, Baharvand H. Efficient differentiation of human embryonic stem cells toward dopaminergic neurons using recombinant LMX1A factor. Mol Biotechnol. 2015; 57(2): 184-194.
- Tahamtani Y, Azarnia M, Farrokhi A, Moradmand A, Mirshahvaladi S, Aghdami N, et al. Stauprimide priming of human embryonic stem cells toward definitive endoderm. Cell J. 2014; 16(1): 63-72.
- Farzaneh Z, Najarasl M, Abbasalizadeh S, Vosough M, Baharvand H. Developing a cost-effective and scalable production of human hepatic competent endoderm from size-controlled pluripotent stem cell aggregates. Stem Cells Dev. 2018; 27(4): 262-274.
- Fathi A, Hatami M, Vakilian H, Han CL, Chen YJ, Baharvand H, et al. Quantitative proteomics analysis highlights the role of redox hemostasis and energy metabolism in human embryonic stem cell differentiation to neural cells. J Proteomics. 2014; 101: 1-16.
- Sahraneshin Samani F, Ebrahimi M, Zandieh T, Khoshchehreh R, Baghaban Eslaminejad M, Aghdami N, et al. In Vitro differentiation of human umbilical cord blood cd133(+) cells into insulin producing cells in co-culture with rat pancreatic mesenchymal stem cells. Cell J. 2015; 17(2): 211-220.
- 13. Atala A, Lanza R, Thomson J, Nerem R. Foundations of Regenerative medicine. San Diego: Elsevier; 2010.
- Ghanian MH, Farzaneh Z, Barzin J, Zandi M, Kazemi-Ashtiani M, Alikhani M, et al. Nanotopographical control of human embryonic stem cell differentiation into definitive endoderm. J Biomed Mater Res A. 2015; 103(11): 3539-3553.
- Murry CE, Keller G. Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development. Cell. 2008; 132(4): 661-680.
- Baharvand H, Mehrjardi NZ, Hatami M, Kiani S, Rao M, Haghighi MM. Neural differentiation from human embryonic stem cells in a defined adherent culture condition. Int J Dev Biol. 2007; 51(5): 371-378.
- Baharvand H, Hashemi SM, Shahsavani M. Differentiation of human embryonic stem cells into functional hepatocyte-like cells in a serum-free adherent culture condition. Differentiation. 2008; 76(5): 465-477.
- Larijani MR, Seifinejad A, Pournasr B, Hajihoseini V, Hassani SN, Totonchi M, et al. Long-term maintenance of undifferentiated human embryonic and induced pluripotent stem cells in suspension. Stem Cells Dev. 2011; 20(11): 1911-1923.
- Vosough M, Omidinia E, Kadivar M, Shokrgozar M-A, Pournasr B, Aghdami N, et al. Generation of functional hepatocyte-like cells from human pluripotent stem cells in a scalable suspension culture. Stem Cells Dev. 2013; 22(20): 2693-2705.
- Nemati S, Abbasalizadeh S, Baharvand H. Scalable expansion of human pluripotent stem cell-derived neural progenitors in stirred suspension bioreactor under xeno-free condition. Methods Mol Biol. 2016; 1502: 143-158.
- Fathi A, Pakzad M, Taei A, Brink TC, Pirhaji L, Ruiz G, et al. Comparative proteome and transcriptome analyses of embryonic stem cells during embryoid body-based differentiation. Proteomics. 2009; 9(21): 4859-4870.
- Bratt-Leal AM, Carpenedo RL, McDevitt TC. Engineering the embryoid body microenvironment to direct embryonic stem cell differentiation. Biotechnol Prog. 2009; 25(1): 43-51.
- Dang SM, Kyba M, Perlingeiro R, Daley GQ, Zandstra PW. Efficiency of embryoid body formation and hematopoietic development from embryonic stem cells in different culture systems. Biotechnol Bioeng. 2002; 78(4): 442-453.
- Yodmuang S, Marolt D, Marcos-Campos I, Gadjanski I, Vunjak-Novakovic G. Synergistic effects of hypoxia and morphogenetic factors on early chondrogenic commitment of human embryonic stem cells in embryoid body culture. Stem Cell Rev. 2015; 11(2): 228-241.
- Gil CH, Lee JH, Seo J, Park SJ, Park Z, Kim J, et al. Well-defined differentiation of HESC-derived hemangioblasts by embryoid body formation without enzymatic treatment. Biotechnol Lett. 2015; 37(6): 1315-1322.
- Dhara SK, Stice SL. Neural differentiation of human embryonic stem cells. J Cell Biochem. 2008; 105(3): 633-640.
- Shin S, Mitalipova M, Noggle S, Tibbitts D, Venable A, Rao R, et al. Long-term proliferation of human embryonic stem cell-derived neuroepithelial cells using defined adherent culture conditions. Stem Cells. 2006; 24(1): 125-138.
- 28. Lund AW, Yener B, Stegemann JP, Plopper GE. The natural and

engineered 3D microenvironment as a regulatory cue during stem cell fate determination. Tissue Eng Part B Rev. 2009; 15(3): 371-380.

- Osafune K, Caron L, Borowiak M, Martinez RJ, Fitz-Gerald CS, Sato Y, et al. Marked differences in differentiation propensity among human embryonic stem cell lines. Nat Biotechnol. 2008; 26(3): 313-315.
- Pal R, Totey S, Mamidi MK, Bhat VS. Propensity of human embryonic stem cell lines during early stage of lineage specification controls their terminal differentiation into mature cell types. Exp Biol Med (Maywood). 2009; 234(10): 1230-1243.
- Lunn JS, Sakowski SA, Hur J, Feldman EL. Stem cell technology for neurodegenerative diseases. Ann Neurol. 2011; 70(3): 353-361.
- Shahbazi E, Moradi S, Nemati S, Satarian L, Basiri M, Gourabi H, et al. Conversion of human fibroblasts to stably self-renewing neural stem cells with a single zinc-finger transcription factor. Stem Cell Reports. 2016; 6(4): 539-551.
- Margolis EB, Coker AR, Driscoll JR, Lemaître AI, Fields HL. Reliability in the identification of midbrain dopamine neurons. PLoS One. 2010; 5(12): e15222.
- Baharvand H, Ashtiani SK, Valojerdi MR, Shahverdi A, Taee A, Sabour D. Establishment and in vitro differentiation of a new em-

bryonic stem cell line from human blastocyst. Differentiation. 2004; 72(5): 224-229.

- Baharvand H, Ashtiani SK, Taee A, Massumi M, Valojerdi MR, Yazdi PE, et al. Generation of new human embryonic stem cell lines with diploid and triploid karyotypes. Dev Growth Differ. 2006; 48(2): 117-128.
- Rassouli H, Tabe Bordbar MS, Rezaei Larijani M, Pakzad M, Baharvand H, Salekdeh GH. Cloning, expression and functional characterization of in-house prepared human basic fibroblast growth factor. Cell J. 2013; 14(4): 282-291.
- Basma H, Soto-Gutierrez A, Yannam GR, Liu L, Ito R, Yamamoto T, et al. Differentiation and transplantation of human embryonic stem cell-derived hepatocytes. Gastroenterology. 2009; 136(3): 990-999.
- Lancaster MA, Renner M, Martin CA, Wenzel D, Bicknell LS, Hurles ME, et al. Cerebral organoids model human brain development and microcephaly. Nature. 2013; 501(7467): 373-379.
- Renner M, Lancaster MA, Bian S, Choi H, Ku T, Peer A, et al. Selforganized developmental patterning and differentiation in cerebral organoids. EMBO J. 2017; 36(10): 1316-1329.
- Enver T, Pera M, Peterson C, Andrews PW. Stem cell states, fates, and the rules of attraction. Cell Stem Cell. 2009; 4(5): 387-397.

Development of An Artificial Male Germ Cell Niche Using Electrospun Poly Vinyl Alcohol/Human Serum Albumin/Gelatin Fibers

Zahra Borzouie, Ph.D.^{1, 2}, Majid Naghibzadeh, M.Sc.², Ali Reza Talebi, Ph.D.², Fatemeh Pourrajab, Ph.D.³, Ali Jebali, Ph.D.⁴, Habib Nikukar, M.D.^{1, 4}, Hosein Molla Hoseini, M.Sc.⁵, Arezoo Khoradmehr, M.Sc.², Fatemeh Sadeghian-Nodoushan, M.Sc.⁶, Behrouz Aflatoonian, Ph.D.^{1, 4, 7*}, Seyedhossein Hekmatimoghaddam, M.D.^{1, 4*}

1. Stem Cell Biology Research Center, Yazd Reproductive Sciences Institute, Shahid Sadoughi University of Medical Sciences, Yazd, Iran 2. Research and Clinical Center for Infertility, Yazd Reproductive Sciences Institute, Shahid Sadoughi University of Medical Sciences, Yazd, Iran

 Department of Biochemistry and Molecular Biology, School of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran
 Department of Advanced Medical Sciences and Technologies, School of Paramedicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran

5. Textile Engineering Faculty, Isfahan University of Technology, Isfahan, Iran

6. Medical Nanotechnology and Tissue Engineering Research Center, Yazd Reproductive Sciences Institute, Shahid Sadoughi University of Medical Sciences, Yazd, Iran

7. Department of Reproductive Biology, School of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran

*Corresponding Address: Stem Cell Biology Research Center, Yazd Reproductive Sciences Institute, Shahid Sadoughi University of Medical Sciences, Yazd, Iran Emails: b.aflatoonian@ssu.ac.ir, shhekmati2002@yahoo.com

Received: 23/June/2018, Accepted: 29/September/2018

Abstract –

Objective: Recent achievements in stem cell biotechnology, nanotechnology and tissue engineering have led to development of novel approaches in regenerative medicine. Azoospermia is one of the challenging disorders of the reproductive system. Several efforts were made for isolation and culture of testis-derived stem cells to treat male infertility. However, tissue engineering is the best approach to mimic the three dimensional microenvironment of the testis *in vitro*. We investigated whether human testis-derived cells (hTCs) obtained by testicular sperm extraction (TESE) can be cultured on a homemade scaffold composed of electrospun nanofibers of homogeneous poly (vinyl alcohol)/human serum albumin/gelatin (PVA/HSA/gelatin).

Materials and Methods: In this experimental lab study, human TCs underwent two steps of enzymatic cell isolation and five culture passages. Nanofibrous scaffolds were characterized by scanning electron microscopy (SEM) and Fourier-transform infrared spectroscopy (FTIR). Attachment of cells onto the scaffold was shown by hematoxylin and eosin (H&E) staining and SEM. Cell viability study using MTT [3-(4, 5-dimethyl-2-thiazolyl) -2, 5-diphenyl -2H- tetrazolium bromide] assay was performed on days 7 and 14.

Results: Visualization by H&E staining and SEM indicated that hTCs were seeded on the scaffold. MTT test showed that the PVA/HSA/gelatin scaffold is not toxic for hTCs.

Conclusion: It seems that this PVA/HSA/gelatin scaffold is supportive for growth of hTCs.

Keywords: Azoospermia, Human Serum Albumin, Scaffold, Testis, Tissue Engineering

Cell Journal(Yakhteh), Vol 21, No 3, October-December (Autumn) 2019, Pages: 300-306 ____

Citation: Borzouie Z, Naghibzadeh M, Talebi AR, Pourrajab F, Jebali A, Nikukar H, Molla Hoseini H, Khoradmehr A, Sadeghian-Nodoushan F, Aflatoonian B, Hekmatimoghaddam S. Development of an artificial male germ cell niche using electrospun poly vinyl alcohol/human serum albumin/gelatin fibers. Cell J. 2019; 21(3): 300-306. doi: 10.22074/cellj.2019.6120.

Introduction

Almost 7% of all men, including those who lack sperm production, suffer from infertility (1). Stem cells, with their great and unique capacity to form other cell types, have raised huge hopes for scientists and clinicians as well as patients with male infertility. After the derivation of mouse embryonic stem cells (2), different studies used mouse primordial germ cells (PGCs) to investigate the biology of germ cells and their progenitors (3, 4). Later, in 1998, the first pluripotent stem cells were generated from pre-implantation human embryos in blastocyst stage (5), and also human PGCs (6) which were named human embryonic stem cells (hESCs) and human embryonic germ cells (hEGCs), respectively. Since 2003, several studies have shown the potential of the ESCs to form male and female germ cells (7-10). However, no gamete has been produced so far. Some investigations made efforts to reprogram unipotent spermatogonial stem cells (SSCs) to derive pluripotent germ-line stem cells (GSCs) in vitro

in mice (11), rats (12) and humans (13). Nonetheless, later reports indicated that human testis-derived cells (hTCs) are not pluripotent and possess characteristics similar to those of mesenchymal stromal cells (14). The latest studies including Irie and Surani's investigations (15) revealed that germ cell development in humans differs from that in mice especially in terms of gene expression profile, which might be the reason for variations in results. Despite improvements in the field, there are still challenges for translation of stem cell biotechnology to bedside practice (i.e. has not yet been used in male reproductive/regenerative medicine).

In a recent study, mouse fertile sperm production from GSCs was done using organ culture (16). Besides developmental differences between mouse and human germ cells, there are more restrictive ethical issues regarding human organ culture compared to mouse organ culture. Therefore, tissue engineering methods are highly required for regeneration of some tissues and organs. These methods prepare bio-scaffolds to promote the development of new tissues such as cartilage or bone. In comparison with other instances where tissue engineering produced artificial tissues, researchers in the field of human male infertility could not obtain adequate mature cells. In regenerative medicine, utilization of pluripotent or multipotent stem cells has higher chance of success compared to unipotent cells like human SSCs (17).

We previously showed the mulipotency of hTCs obtained from TESE samples (14). The aim of this study was to make a homemade scaffold composed of electrospun fibers of homogeneous solution of poly (vinyl alcohol)/ human serum albumin/gelatin (PVA/HSA/gelatin) as a niche for hTCs. Development of an artificial organ culture for production of male germ cells from hESC-derived GSCs could be the ultimate goal in this field.

Materials and Methods

Fabrication of the scaffold

In this experimental lab study, initially 450 mg of PVA powder (Merck, Germany, MW 72,000) was dissolved in deionized water (to reach a final concentration of 7% w/v) in a final volume of 6 mL which was kept at 80°C for 5 hours in a sterile beaker to make a clear solution. Next, 0.3 g gelatin powder (Merck, Germany) was added and the mixture was mixed by a magnetic stirrer at room temperature (RT). Then, 2 mL of a 20 g/dL solution of HSA (CSL Behring AG, Switzerland) was added to the mixture and mixed for 60 minutes on a magnetic stirrer. The resulting solution was homogenous and milky white.

The prepared homogeneous PVA/HSA/gelatin solution was electrospun into fibers using Electroris (FNM Ltd., Iran). The instrument consisted of a high voltage power supply, a conductive collector, a reservoir of polymer solution, and a nozzle with adjustable distance to collector.

To produce electrospun fibers, the polymer solution was drawn into a 5 mL syringe with a metallic needle of 0.4 mm internal diameter. The syringe was kept horizontally on the syringe stand with the metal needle tip being connected to the positive electrode of the high voltage power supply. The voltage was set at 16 KV, and the distance from collector was 10 cm. The experiment was done at RT (25° C) (18). The fibers were collected after 3 hours on circular glass coverslips. The obtained PVA/albumin/gelatin fibrous scaffold was further cross-linked in glutaraldehyde vapor at RT for 1 day, then immersed in deionized water to remove the glutaraldehyde. The cross-linked scaffolds were dried and prepared for testicular cells culture (19).

Chemical analysis of scaffolds

Fourier-transform infrared (FTIR) spectroscopy conducted over a range of 4000-500 cm⁻¹ was used for analysis of the PVA/HSA/gelatin fibrous scaffolds. The Nicolet spectrometer system (BOMEM FTIR MB-series, MB-100, Hartmann & Braun, Canada) provided FTIR spectra using a DTGS KBr detector. For this, about 1 mg of dried scaffold was mixed with 100-120 mg of KBr to make compressed pellets.

Determination of scaffolds' hydrophilicity, morphology, fiber diameter and pore size

Before and after exposure to glutaraldehyde, water contact angles of electrospun scaffold were measured by a video-based optical system (model MV500 digital microscope, EasyTear, Italy). The images of water drops on the PVA/HSA/gelatin scaffold surface from three different angles were captured by the camera and analyzed by Digimizer image analysis software (MedCalc Software bvba, Belgium) to assess hydrophilicity. The volume of each water droplet was 5 μ L, and measurements were done 10 seconds after contact.

To evaluate the attachment of hTCs onto fibers, we performed hematoxylin (Merck, Germany) and eosin (Merck, Germany) (H&E) staining on scaffolds, on glass slides on days 7 and 14.

The morphology of the scaffold was also characterized by scanning electron microscopy (SEM, model Phenom ProX, Phenom-World, The Netherlands) with an accelerating voltage of 15 kV after coating with gold. The average diameter of fibers and pore sizes were randomly determined by image analysis software (ImageJ, National Institute of Health, USA) to analyze 100 different fibers in each SEM image.

Sample collection and patients' information

TESE samples were collected after obtaining signed informed consent from two patients with non-obstructive azoospermia attending a clinic for assisted reproduction. This study was approved by Ethics Committee of Shahid Sadoughi University of Medical Sciences, Yazd, Iran with reference No. IR.SSU.REC.1394.226. These two patients were chosen because their biopsies proved to contain germ cells. The fresh samples (about 40 mg each) were labeled with codes to maintain patient anonymity, placed in 2 mL of Dulbecco's Modified Eagle Medium containing 5% fetal bovine serum (DMEM/5% FBS) (Invitrogen, UK), and transferred to the laboratory within 15 minutes (14).

Preparation of human testis-derived cells from TESE samples

Approximately 30-40 mg pieces of the TESE samples were washed in DMEM medium and mechanically and enzymatically [collagenase type IV (Invitrogen, UK)] digested overnight using a previously reported protocol (14). The cells were subsequently recovered by aspiration, washed with DMEM and centrifuged for 3 minutes at 200 g. The supernatant was discarded, and the pellet was used for hTCs culture.

Culture of human testis-derived cells

The initial culture protocol was previously described by Sadeghian-Nodoushan et al. (14). Single-cell suspensions were placed in dishes with 45 mL of DMEM supplemented with 5 mL FBS, 100 ng/mL glial cell-derived neurotrophic factor (GDNF, R&D Systems, USA), and 20 ng/mL epidermal growth factor (EGF, R&D Systems, USA). Most of the testicular cells were attached to the dish floor the day after initial extraction, and about 50% of the culture medium was exchanged every other day. Enzymatic treatment using trypsin (Sigma, Germany), and EDTA (Invitrogen, UK) was performed at 37°C for 3 minutes for passaging the hTCs. All cell culture experiments were performed at least in triplicate.

Transfer of human testis-derived cells on scaffold

The scaffolds were sterilized by one-hour UV irradiation. After five passages of hTCs in dishes, the cells were disaggregated using trypsin/EDTA, enumerated using a hemacytometer slide, plated on the scaffold at a concentration of 5×10^3 cells/coverslip placed in sterile dishes, and maintained at 34°C with 5% CO₂. The cell-coated scaffolds were checked for cell proliferation/viability by the MTT [3-(4, 5-dimethyl-2-thiazolyl) -2, 5-diphenyl -2H- tetrazolium bromide] test on days 7 and 14.

Cell viability and proliferation assay (MTT assay)

To evaluate the viability and proliferation rate of the hTCs on the scaffold, we used the MTT test as a standard colorimetric assay which assesses cell viability based on the mitochondrial dehydrogenase activity. Briefly, on days 7 and 14, following cell incubation with and without scaffolds, 40 μ L of MTT solution (5 mg/mL in RPMI) was added to each central well (containing coverslips covered by the scaffold containing mixed testicular cells); then, the supernatant was removed, and 400 μ L of 0.1 M HCl (prepared in isopropyl alcohol) was added to dissolve formazan crystals. The optical densities (OD) at 570 nm (with background subtraction at 630 nm) were evaluated using an ELISA (enzyme-linked immunosorbent assay) reader (Tajhizat Sanjesh, Iran). Percentage of viability and proliferation was determined by the following formula:

Percentage of viability= Optical density (OD) of the test sample/OD of the control sample×100

Any proliferation or decrease in the number of cells in scaffolds would so have been evident from their OD. All experiments were done in triplicate and the mean of three replicates were reported.

Statistical analysis

The student's t test was used for comparison of mean of proliferation and viability between the contro (monolayer) and experimental (culture on scaffold) groups. The SPSS software version 16 (IBM SPSS Statistics, USA) was used for statistical analysis. Any P<0.05 was considered indicative of significant difference between groups.

Results

Fabrication and characterization of fibrous scaffolds

A PVA/HSA/gelatin homemade fibrous scaffold was designed by electrospinning for hTCs culture. Surface structure of composite fibers is shown in Figure 1A. Fiber diameter was 100-600 nm (mean diameter 305 nm) (Fig.1B), and the average of pore sizes was 0.810 µm (Fig.1C). Surface wettability as an important determinant of cell adhesion,

proliferation, and migration, was also checked. The scaffolds were found to be hydrophilic with contact angles of 28.2° and 46.8°, before and after cross-linkage in glutaraldehyde vapor, respectively. Contact angle data supported the hypothesis that incorporation of glutaraldehyde into scaffolds decreases hydrophilicity which consequently leads to higher biostability.



Fig.1: Physical analysis of electrospun scaffold. **A.** Scanning electron micrograph of poly (vinyl alcohol)/human serum albumin/gelatin (PVA/HSA/gelatin) fibers (scale bar: 1 µm), **B.** Fiber diameter distribution of PVA/HSA/gelatin scaffold, and **C.** Fiber pore diameters of PVA/HSA/gelatin scaffold.

Fourier-transform infrared spectroscopy spectra

Chemical analysis of fibrous scaffold showed typical spectrum peaks for PVA, HSA and gelatin (Fig.2). The result for PVA showed absorption peaks at about 3200-3550 cm⁻¹ (OH-OH stretching), 2930 cm⁻¹ (C-H stretching), 1245 cm⁻¹ (C-O stretching), 1084 cm⁻¹ (C-O)-C-OH stretching), 937 cm⁻¹ (CH-CH, stretching) and 865 cm⁻¹ (C-C stretching). The result for gelatin showed absorption peaks at 1640-1636 cm⁻¹ (amide I), 1544-1542 cm⁻¹ (amide II), 1240 cm⁻¹ (amide III) and 3300 cm⁻¹ (amide A). The FTIR spectrum for HSA showed strong absorption peaks at 1550 cm⁻¹ (amide I) and 1660 cm⁻¹ (amide II). In the PVA/HSA/ gelatin fibers, very clear absorption peaks assigned to the PVA, were present at OH-OH and C-H stretching bands. Further typical absorptions were seen at amide I and amide II which can be assigned to the HSA as well as gelatin. These results indicated the presence of all three materials in the fiber.



 $\ensuremath{\mbox{Fig.2:}}$ Fourier transform infrared spectroscopy of the scaffold and its constituents.

Isolation and culture of human testis-derived cells

During assessment of the spermatogenesis status of TESE samples, histological analysis of testicular tissue demonstrated the presence of somatic and germ cells in the tissue. Isolation of germ cells was not the aim of this study; we required just a few SSCs in the tissue as germ cells harboring stemness potential documented by H&E staining.

The hTCs were initially floating, but began to attach after culture in the central dish. After one week, many of the cells were adherent and began to grow. After 5 passages, we had adequate numbers of cells to continue the study.

Morphology of human testis-derived cells

The presence of cells on scaffold was proved by SEM (not shown here). The results showed that the scaffold had the ability to support the hTCs during 14 days. Cells attachment to fibers and their normal shape were also demonstrated by H&E staining (Fig.3). It seems that this scaffold can mimic extracellular matrix (ECM).



Fig.3: H&E staining of 7-day cultured cells seeded on poly (vinyl alcohol)/ human serum albumin/gelatin fibers (scale bar: 200 µm).

Table 1: Comparison of viability between testis cells cultured on scaffold and monolayer-cultured ones							
Parameters	Optical density (7 th day)	Viability (7 th day)	Optical density (14th day)	Viability (14 th day)			
Group							
Monolayer	0.20 ± 0.1	-	0.23 ± 0.07	-			
Electrospun scaffold	0.17 ± 0.06	85	0.18 ± 0.06	78.26			

Data are presented as mean ± SD (for optical density, triplicate) and percentage (for viability).

Proliferation and viability of human testis-derived cells

Table 1 shows the percentages of viable cells after 7 and 14 days of culture in both monolayer and scaffold-cultured groups treated with growth factors GDNF and EGF. About 85% of cells cultured on scaffold were viable on day 7 (in comparison with the monolayer control group which showed 100% viability), with a small drop in this figure at day 14 (78.26%). There was no significant difference (Student's t test, P>0.05) in cell viability and proliferation rate between the control group and experimental groups based on MTT test results. Our data suggest the nontoxic nature of this scaffold for hTCs.

Discussion

SSCs play crucial roles as male gamete (sperm) precursor cells which transfer father's genetic information to the next generation. They are unipotent stem cells and their population in the testis is very small (20). Recently, in vitro production of haploid cells from SSC-like cells was shown in mice (21). Interestingly, in vitro production of functional sperms was confirmed by other studies using organ culture of SSC lines in neonatal mouse testis (22, 23). Nonetheless, there are ethical and practical challenges to achieve this aim in humans. Firstly, getting neonatal human testis biopsies to grow human SSCs is almost impossible. Secondly, despite the efforts made to isolate and expand human SSCs in culture to generate GSCs or human testisderived embryonic-like stem cells (htESC-like cells) (13, 24, 25), some reports have indicated that these hTCs are not pluripotent and possess multipotent stromal characteristics (14).

It was shown in a study that induced pluripotent stem (iPS) cell-derived cells injected along with testicular cells into dorsal skin of mice are able to reconstitute seminiferous tubules, and iPS cell-derived germ cells can lodge at basement membranes of reconstituted tubules (26).

Tissue engineering methods using stem cells are applicable strategies in some problematic cases (27, 28). These methods can be used, for example, for synthesis of artificial ECM as a niche for cells in culture (29-31). *In vivo*, the SSCs are connected in some ways to other cells such as Sertoli and Leydig cells. This close proximity is very important for cells to exchange signals through secretion of growth factors (32). The scaffold should provide better conditions for the cells similar to those present in *in vivo* 3D condition (33).

Previously, we showed construction of an artificial human testis using homemade human serum albumin and calcium phosphate 3D scaffolds coated with hTCs. Although histological structures similar to human seminiferous tubules were formed, but their arrangement was not comparable to that of the cells within the human testis (34).

Electrospinning is an applicable method used in drug delivery and tissue engineering. Different types of materials like poly (lactic acid)/chitosan, and PVA, have been used to make micro/nanofibers (18, 35-39). In this study, human testicular cells were seeded on an electrospun PVA/HSA/gelatin fibrous mesh, to develop a 3D niche suitable for human male germ cells. HSA is the most abundant protein in human serum (35-50 g/L) with half-life of about 19 days. HSA was selected in this study because it is a very soluble globular monomeric protein besides being stable in the pH range of 4-9 and at high temperatures which is very critical in the process of making nanofibers. Temperature stability at near 60°C for up to 10 hours is necessary in this method. Another advantage is that when HSA is broken down, the resulting amino acids will nourish surrounding tissues. HSA is not only very cheap but also quite available. Finally, HSA has no toxicity and is biodegradable, two important points in regenerative medicine (40). Compared to the work on rat testicular cells seeded on poly(D,L-lacticco-glycolic acid) porous scaffolds which showed promising 75% viability up to 18 days and some degree of differentiation (39), our study on human testicular cells yielded 78% viability on the 14th day.

In the present experiment, the initial number of cells cultured on each scaffold was 5000 cells. Since the supernatant of each microplate well was used for the MTT test, the optical densities reflect the number of cells. Since enumeration of the cells present on each scaffold was not easy, the only indicator of any proliferation or decrease in cell counts was the OD.

Since the OD of wells containing cells cultured on scaffolds were not significantly different from that of the monolayer cultures (used as the control group), we may conclude that they have proliferated only a little less than cells on the monolayer culture.

In our study, the viability and proliferation of the cells were examined by MTT assay and results indicated that this device is not toxic for the cells. SEM images showed homing of the cells within the fibers. Our data may serve as the starting point of human ambitions for recapitulation of human testis and probably other organs, with conceivable further applications in human developmental biology, toxicology, drug discovery and regenerative medicine.

Conclusion

In this study, a novel PVA/HSA/gelatin fibrous scaffold was designed and tested for physical, chemical and biologic properties, including its toxicity for hTCs. Promising performance of this scaffold in terms of biocompatibility and support of hTC growth encourages further evaluation of its *in vivo* ability to induce sperm production in animal models and then in human experiments.

Acknowledgements

The authors thank Dr. Vahidi for provision of testis biopsies from his azoospermic patients. Ms Afzali is also acknowledged for data analysis/software works. Financial support was provided by Shahid Sadoughi University of Medical Sciences, Yazd, Iran. The authors declare that there is no conflict of interest.

Authors' Contributions

Z.B.; Performed the majority of tests and procedures, interpretation of the data and the conclusion. M.N.; Helped with electrospinning and nano work. A.R.T.; Helped with testis tissue handling, and consulted on germ cell acquisition. F.P.; Gave consultation about study design and conception, and drafted the manuscript. A.J.; Assisted with nanomaterial preparations and gave consultation on data gathering. H.N.; Assisted with nanomaterial preparation, and gave consultation on tissue scaffolds. H.M.H.; Helped with contact angle determination, electrospinning and gelatin fiber preparation. A.K.; Provision of lab ware and general test assistance. F.S.-N.; Helped with cell cultures, provided materials and collected data. B.A.; Gave consultation about the whole study, helped with cell culture and imaging. S.H.; Designed and supervised the study, performed the final revision of the manuscript and is responsible for scientific integrity of the article. All authors read and approved the final manuscript, and took responsibility over its contents.

References

- Louis JF, Thoma ME, Sorensen DN, McLain AC, King RB, Sundaram R, et al. The prevalence of couple infertility in the United States from a male perspective: evidence from a nationally representative sample. Andrology. 2013; 1(5): 741-748.
- Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. Nature. 1981; 292(5819): 154-156.
- De Felici M, McLaren A. In vitro culture of mouse primordial germ cells. Exp Cell Res. 1983; 144(2): 417-427.
- Matsui Y, Zsebo K, Hogan BL. Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. Cell. 1992; 70(5): 841-847.
- Thomson JA, İtskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, et al. Embryonic stem cell lines derived from human blastocysts. Science. 1998; 282(5391): 1145-1147.
- Shamblott MJ, Axelman J, Wang S, Bugg EM, Littlefield JW, Donovan PJ, et al. Derivation of pluripotent stem cells from cultured human primordial germ cells. Proc Natl Acad Sci USA. 1998; 95(23): 13726-13731.
- Hübner K, Fuhrmann G, Christenson LK, Kehler J, Reinbold R, De La Fuente R, et al. Derivation of oocytes from mouse embryonic stem cells. Science 2003; 300(5623): 1251-1256.
- Clark AT, Bodnar MS, Fox M, Rodriquez RT, Abeyta MJ, Firpo MT, et al. Spontaneous differentiation of germ cells from human embryonic stem cells in vitro. Hum Mol Genet. 2004; 13(7): 727-739.
- Aflatoonian B, Moore H. Human primordial germ cells and embryonic germ cells, and their use in cell therapy. Curr Opin Biotech. 2005; 16(5): 530-535.
- Aflatoonian B, Ruban L, Jones M, Aflatoonian R, Fazeli A, Moore HD. In vitro post-meiotic germ cell development from human embryonic stem cells. Hum Reprod. 2009; 24(12): 3150-3159.
- 11. Kanatsu-Shinohara M, Inoue K, Lee J, Yoshimoto M, Ogonuki N, Miki H, et al. Generation of pluripotent stem cells from neo-

natal mouse testis. Cell. 2004; 119(7): 1001-1012.

- Ryu BY, Orwig KE, Kubota H, Avarbock MR, Brinster RL. Phenotypic and functional characteristics of spermatogonial stem cells in rats. Dev Biol. 2004; 274(1): 158-170.
- Mizrak SC, Chikhovskaya JV, Sadri-Ardekani H, van Daalen S, Korver CM, Hovingh SE, et al. Embryonic stem cell-like cells derived from adult human testis. Hum Reprod. 2010; 25(1): 158-167.
- Sadeghian-Nodoushan F, Aflatoonian R, Borzouie Z, Akyash F, Fesahat F, Soleimani M, et al. Pluripotency and differentiation of cells from human testicular sperm extraction: An investigation of cell stemness. Mol Reprod Dev. 2016; 83(4): 312-323.
- Irie N, Surani MA. Efficient induction and isolation of human primordial germ cell-like cells from competent human pluripotent stem cells. Method Mol Biol. 2017;1463: 217-226.
- Sato T, Katagiri K, Kubota Y, Ogawa T. In vitro sperm production from mouse spermatogonial stem cell lines using an organ culture method. Nat Protoc. 2013; 8(11): 2098-2104.
- Ringe J, Kaps C, Burmester GR, Sittinger M. Stem cells for regenerative medicine: advances in the engineering of tissues and organs. Naturwissenschaften. 2002; 89(8): 338-351.
- Won JJ, Nirmala R, Navamathavan R, Kim HY. Electrospun core-shell nanofibers from homogeneous solution of poly (vinyl alcohol)/bovine serum albumin. Int J Biol Macromol. 2012; 50(5): 1292-1298.
- Li Y, Chen F, Nie J, Yang D. Electrospun poly (lactic acid)/chitosan core–shell structure nanofibers from homogeneous solution. Carbohydr Polym. 2012; 90(4): 1445-1451.
- Dym M, Kokkinaki M, He Z. Spermatogonial stem cells: mouse and human comparisons. Birth Defects Res C Embryo Today. 2009; 87(1): 27-34.
- 21. Wang P, Suo LJ, Shang H, Li Y, Li GX, Li QW, et al. Differentiation of spermatogonial stem cell-like cells from murine testicular tissue into haploid male germ cells in vitro. Cytotechnology. 2014; 66(3): 365-372.
- Sato T, Katagiri K, Gohbara A, Inoue K, Ogonuki N, Ogura A, et al. In vitro production of functional sperm in cultured neonatal mouse testes. Nature. 2011; 471(7339): 504-507.
- 23. Sato T, Katagiri K, Kubota Y, Ogawa T. In vitro sperm production from mouse spermatogonial stem cell lines using an organ culture method. Nat Protoc. 2013; 8(11): 2098-2104.
- Kossack N, Meneses J, Shefi S, Nguyen HN, Chavez S, Nicholas C, et al. Isolation and characterization of pluripotent human spermatogonial stem cell-derived cells. Stem Cells. 2009; 27(1): 138-149.
- Sadri-Ardekani H, Mizrak SC, van Daalen SK, Korver CM, Roepers-Gajadien HL, Koruji M, et al. Propagation of human spermatogonial stem cells in vitro. JAMA. 2009; 302(19): 2127-2134.
- Yang S, Bo J, Hu H, Guo X, Tian R, Sun C, et al. Derivation of male germ cells from induced pluripotent stem cells in vitro and in reconstituted seminiferous tubules. Cell Prolif. 2012; 45(2): 91-100.
- Caplan AI. Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. J Cell Physiol. 2007; 213(2): 341-347.
- Barrilleaux B, Phinney DG, Prockop DJ, O'connor KC. Review: ex vivo engineering of living tissues with adult stem cells. Tissue Eng. 2006; 12(11): 3007-3019.
- Badylak SF. The extracellular matrix as a scaffold for tissue reconstruction. Semin Cell Dev Biol. 2002; 13(5): 377-383.
- Li WJ, Laurencin CT, Caterson EJ, Tuan RS, Ko FK. Electrospun nanofibrous structure: a novel scaffold for tissue engineering. J Biomed Mater Res. 2002; 60(4): 613-621.
- Lutolf MP, Hubbell JA. Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. Nat Biotechnol. 2005; 23(1): 47-55.
- Clermont Y. Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. Physiol Rev. 1972; 52(1): 198-236.
- Saltzman WM, Parkhurst MR, Parsons-Wingerter P, Zhu WH. Three-dimensional cell cultures mimic tissues. Ann NY Acad Sci. 1992; 665: 259-273.
- 34. Borzouie Z, Hekmati-Moghadam SH, Talebi AR, Poor-Rajab F, Jebali A, Nikukar H, et al. Reconstitution of an artificial human

testis using a 3 dimensional (3D) culture device. Proceedings of the 31st Annual Meeting of the European Society for Human Reproduction and Embryology, Lisbon. Hum Reprod. 2015; 30 Suppl 1: P-788

- Sill TJ, von Recum HA. Electrospinning: applications in drug delivery and tissue engineering. Biomaterials. 2008; 29(13): 1989-2006.
- Li Y, Chen F, Nie J, Yang D. Electrospun poly (lactic acid)/chitosan core–shell structure nanofibers from homogeneous solution. Carbohydr Polym. 2012; 90(4): 1445-1451.
- Zhang C, Yuan X, Wu L, Han Y, Sheng J. Study on morphology of electrospun poly (vinyl alcohol) mats. Eur Polym J. 2005; 41(3): 423-432.
- Eslahi N, Hadjighassem MR, Joghataei MT, Mirzapour T, Bakhtiyari M, Shakeri M, et al. The effects of poly L-lactic acid nanofiber scaffold on mouse spermatogonial stem cell culture. Int J Nanomedicine. 2013; 8: 4563-4576.
- Lee JH, Oh JH, Lee JH, Kim MR, Min CK. Evaluation of in vitro spermatogenesis using poly (D, L-lactic-co-glycolic acid)(PLGA)based macroporous biodegradable scaffolds. J Tissue Eng Regen Med. 2011; 5(2): 130-137.
- Elzoghby AO, Samy WM, Elgindy NA. Albumin-based nanoparticles as potential controlled release drug delivery systems. J Control Release. 2012; 157(2): 168-182.

Stress Hormone and Oxidative Stress Biomarkers Link Obesity and Diabetes with Reduced Fertility Potential

Shima Abbasihormozi, Ph.D.^{1, 2}, Vahab Babapour, Ph.D.¹, Azam kouhkan, M.D.^{3, 4}, Amir Niasari Naslji, Ph.D.⁵, Kaveh

Afraz, M.Sc.⁶, Zahra Zolfaghary, M.Sc.³, Abdolhossein Shahverdi, Ph.D.^{2, 3*}

1. Department of Basic Science, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran 2. Department of Embryology, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran

3. Reproductive Epidemiology Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran 4. Department of Regenerative Biomedicine, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

5. Department of Midwifery and Reproductive Diseases, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran 6. Department of Andrology, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran

*Corresponding Address: P.O.Box: 16635-148, Reproductive Epidemiology Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran Email: shahverdi@royaninstitute.org

Received: 1/September/2018, Accepted: 11/December/2018 Abstract

Objective: Tilting the balance in favor of antioxidant agents could increase infertility problems in obese and diabetic individuals. The aim of this study was to evaluate oxidative stress status in semen of men with type 2 diabetes and obesity to investigate whether excessive amounts of oxidative stress, as a result of diabetes and obesity, influence infertility potential.

Materials and Methods: A case-control study was conducted in men (n=150) attending the Infertility Center of Royan Institute between December 2016 and February 2017. Participants were categorized into four groups; normal weight (BMI<25 kg/m²) and non-type-2 diabetic (control=40), obese and non- type-2 diabetic (obese=40), non-obese and type-2 diabetic (Nob-DM=35), and obese and type-2 diabetic (Ob-DM=35). The semen analysis was performed according to the World Health Organization criteria. Oxidative stress, DNA fragmentation, sperm apoptosis, and total antioxidant capacity (TAC) were evaluated in semen samples of men. Serum glucose, HbA1c, cortisol, and testosterone levels were determined using the enzyme-linked immunosorbent assay (ELISA) method.

Results: Compared with the control group, sperm motility, progressive motility, and normal morphology were significantly decreased in the obese, Nob-DM, and Ob-DM groups (P<0.01). The obese, Nob-DM, and Ob-DM groups showed significantly lower levels of TAC and higher amounts of oxidative stress, early apoptotic sperm, and the percentage of DNA fragmentation as compared with the control group (P<0.05). Testosterone concentration was decreased in the obese, Nob-DM, and Ob-DM groups when compared with healthy individuals (P<0.05), whereas the cortisol level was significantly increased in the Nob-DM and Ob-DM groups in comparison to the obese and control group (P<0.01).

Conclusion: Increased amount of reactive oxygen species (ROS) levels and DNA fragmentation in men affected by either diabetes or obesity could be considered prognostic factors in sub-fertile patients, alerting physicians to an early screen of male patients to avoid the development of infertility in prone patients.

Keywords: Antioxidants, Diabetes, Male Infertility, Obesity, Reactive Oxygen Species

Cell Journal(Yakhteh), Vol 21, No 3, October-December (Autumn) 2019, Pages: 307-313

Citation: Abbasihormozi Sh, Babapour V, kouhkan A, Niasari Naslji A, Afraz K, Zolfaghary Z, Shahverdi AH. Stress hormone and oxidative stress biomarkers link obesity and diabetes with reduced fertility potential. Cell J. 2019; 21(3): 307-313. doi: 10.22074/cellj.2019.6339.

Introduction

Diabetes mellitus (DM) and obesity are categorized as metabolic disorders characterized by the presence of chronic hyperglycemia (1) and excessive accumulation of fat in adipose tissue (2). Obesity and diabetes are fast growing problems that they could reach in pandemic proportions in the near future; both metabolic disorders can inevitably affect men in their reproductive age (3). The effect of diabetes and obesity on sperm count, motility, and morphology in humans is controversial. Several pathogenic processes are involved in the development of subfertility in obese and diabetic men (4-6). The possible mechanism underlying diminished reproductive performance in obese men is the generation of the excessive amounts of reactive oxygen species (ROS). The elevated level of ROS is thought to be a risk factor for the development of approximately half of the male infertility cases in men diagnosed with sperm dysfunction (6-10).

Increased levels of circulating glucose and lipids could result in an excessive supply of energy substrates to metabolic pathways in adipose and non-adipose cells which, in turn, can increase the production of ROS (11). Oxidative stress is functionally linked to both glucocorticoid (GC) and immune function (12, 13). It was also reported that elevated levels of GC increase oxidative stress via an elevation of metabolic reactions, causing an increased flux of electrons at the level of the electron transport chain, which could directly oppress reproduction through influencing the hypothalamicpituitary-gonadal axis (14). Notably, there is a lack of sufficient information regarding the relationship between common metabolic disorders, such as obesity and diabetes, and excessive generation of ROS-mediated oxidative stress which may play a key role in the disruption of the hypothalamicpituitary-adrenal (HPA) axis, hypothalamic-pituitarygonad (HPG) axis, and sperm functionality. Therefore, we evaluated the status of oxidative stress, apoptotic cells, DNA fragmentation and total capacity antioxidant as well as the levels of cortisol and testosterone in individuals who were affected by diabetes and obesity.

Materials and Methods

Study design

The case-control study was conducted in men who referred to the Infertility Center of Royan Institute between December 2016 to February 2017. The study was approved by the Ethical and Research Committee of Royan Institute (No.IR.ACECR.ROYAN.REC.1396.53). All participants signed written informed consents. Participants were also asked to fill in a lifestyle questionnaire concerning their lifestyle including information about height, weight, smoking status, alcohol use, and substance abuse, taking any medications, and past medical and surgical history. Weight and height were applied to calculate the body mass index (BMI) according to the following formula: kg/m² where kg is an individual's weight in kilograms and m² is his/her height in meters squared. World Health Organization (WHO) described overweight when a patient possesses (BMI) $\geq 25 \text{ kg/m}^2$; obese when a patient has the BMI \geq 30 kg/m². All participants were classified into four groups as follows: group I: 40 individuals with BMI <25 kg/m² who were non-diabetic mellitus and nonobese (control), group II: 40 subjects with BMI \geq 30 kg/ m² who were obese- non-diabetic mellitus (obese), group III: 35 individuals with BMI \leq 30 kg/m² who were nonobese- diabetic mellitus (Nob-DM), and group IV35 : subjects with BMI \geq 30 kg/m² who were affected by both diabetes and obesity (Ob-DM).

The exclusion criteria were cryptorchidism, testicular varicocele, gonadal disease or abnormality, genital infections, chronic medical disorders such as hypertension, testicular tumors, systemic diseases, and continuous exposure to chemical or physical agents with known adverse reproductive effects.

Labratory tests

Venous blood samples were drawn between 9:00 A.M and 10:00 A.M, after overnight fasting (at least 12 hours). Samples were centrifuged for 15 minutes at 3000 rpm to separate serum specimens.

Serum glucose and glycosylated hemoglobin

Serum glucose was analyzed using a standard enzymatic method (Roche Diagnostics GmbH, Germany). Glycosylated hemoglobin (HbA1c) was quantified using a Nyco Card Reader II analyzer according to the manufacturer's instructions.

Testosterone and cortisol

Serum total testosterone was determined by using a commercially available kit for human total testosterone (TT) ELISA kit (AccuBind ELISA Microwells, Monobina Inc., Lake Forest, CA, USA). Serum cortisol levels were determined by chemiluminescent immunoassays (ADVIA Centaur, USA).

Semen collection and analysis

Semen samples were collected by masturbation after a recommended 3-5 days of sexual abstinence. After semen collection, semen samples were allowed to liquefy at room temperature for at 37°C for 30 minutes before further analysis. Basic sperm parameters including sperm count, concentration, motility, and morphology were evaluated according to the WHO 2010 guidelines (15). After semen liquefaction, sperm progressive motility, normal sperm morphology, semen volume, semen pH, and sperm density were analyzed. Semen volume was also measured. The CASA system [SPERM CLASS ANALYZER software (SCATM, Microptic, Version 4.2, Spain)] was employed to assess progressive sperm motility and sperm concentration. Sperm was classified as progressive motile (WHO class A+B), non-progressive motile (class C) or immotile (class D). Papanicolaou staining was utilized to assess sperm morphology (16).

Measurement of sperm reactive oxygen species

ROS levels were determined in semen specimens following the instructions recommended by the WHO laboratory manual for the examination and processing of human semen. Sperm fresh were washed in a Krebs-Ringer medium (KRM) and adjusted to a concentration of 10 million sperm per milliliter. Chemiluminescent probes, including luminol, formyl-methionyl-leucylphenylalanine (FMLP), and Phorbol 12 myristate 13-acetate (PMA) were utilized to detect ROS generation by white blood cells (WBCs) and sperm. Chemiluminescent signals were monitored using a luminometer (SynergyTM H4 Hybrid Multi-Mode Microplate Reader, BioTek®, USA), and the final ROS level was calculated as relative light units (RLU)/sec/million sperms (17).

Annexin V/PI Assay

AnAnnexinV-FITCApoptosisDetectionKit(Groningen, Netherlands) was used to detect the translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane. Sperm samples were diluted to 1×10^6 sperm/mL in 100 µL of calcium buffer with 5 µg annexin V which was maintained for 15 minutes at room temperature. The samples were simultaneously stained with 10 µg/mL propidium iodide. Among spermatozoa population, late apoptotic spermatozoa were stained with PI, but not with Annexin V whereas early apoptotoic spermatozoa were labeled with both Annexin V and PI. Viable spermatozoa were stained by neither Annexin V nor PI, while apoptotic spermatozoa were labeled only by Annexin V, but not by PI (18)

Assessment of total antioxidant capacity

Total antioxidant capacity (TAC) was measured by colorimetry using a total antioxidant assay kit (MBL, Germany). TAC was analyzed by means of a Microplate Reader (SynergyTM H4 Hybrid Multi-Mode Microplate Reader, BioTek®, USA) and calculated as nmol/µl of semen. Briefly, frozen seminal plasma was thawed by placing vials into a water bath at 37°C for 20 minutes, and immediately assessed for its antioxidant capacity following the manufacturer's instructions (17).

Sperm chromatin structure assay

Sperm chromatin structure assay (SCSA) is a flow cytometry technique, which measures the susceptibility of sperm DNA to acid-induced DNA denaturation in situ. Briefly, frozen semen samples were quickly liquefied in a water bath at 37° C and diluted to a concentration of $1-2\times10^{6}$ sperm per milliliter. Then, 200 µL of the obtained suspension was treated with 200 µL acid-detergent solution for 30 seconds. Afterwards, 900 µL of acridine orange (AO) staining solution (Sigma-Aldrich, St. Louis, MO, USA) was added, and the sample was analyzed by flow cytometry. For each sample, a total of 10,000 events were measured at a flow rate of approximately 200 cells/ sec. DNA fragmentation index (DFI) as a measurement of a degree of sperm DNA damage was identified by the

reflection of red fluorescence to total fluorescence and DFI values, subsequently analyzed using the FlowJo software (19).

Statistical analysis

The analysis of the data was performed using SPSS version 16 (SPSS Inc., Chicago, IL, USA). To test whether the data were normally distributed, Kolmogorov-Smirnov test was carried out, and appropriate statistical tests, either parametric (One-way ANOVA followed by Dunnett's T3 post hoc test) or non-parametric (Kruskal-Wallis test followed by Bonferroni Correction post hoc test) were performed. Correlations between the lipid profiles, sperm parameters, and inflammatory factors were examined by Pearson and Spearman correlation. The p-value of less than 0.05 was statistically significant.

Results

General data

The demographic and clinical characteristics of all individuals who participated in our study are summarized in Table 1. The Nob-DM and Ob-DM groups were significantly (P<0.001) older than other groups. There were significant differences observed between the control and other groups in terms of the mean waist circumference (WC), hip circumference (HC), and waist-to-hip ratio (WHR). Basically, groups comprising diabetic patients had significantly higher fasting blood glucose (FBG) and HbA1C levels as compared with the control and obese groups (P<0.05).

Table 1: Demographic characteristics of all individuals participated in the study. The numbers are adjusted in compliance with age, and BMI

Variable	Groups					
	Control	Obese	Nob-DM	Ob-DM	P value	
Clinical	n=40	n=40	n=35	n=35		
Age (Y)	$33\pm0.97^{\rm a}$	$33\pm0.97^{\rm a}$	$39\pm1.05^{\text{bA}}$	$38.5\pm0.97^{\rm A}$	0.001	
BMI (kg/m ²)	$23.3\pm0.21^{\rm abc}$	$36\pm0.80^{\rm Ab}$	$25.8\pm0.40^{\mathrm{abC}}$	$34\pm0.68^{\rm aB}$	0.001	
Waist (cm)	$88.5\pm0.73^{\rm ab}$	$116\pm1.92^{\rm AB}$	96 ± 1.32^{ab}	$113\pm1.70^{\rm AB}$	0.002	
Hip circumference (cm)	$96.5\pm0.68^{\rm a}$	$112\pm3.18^{\scriptscriptstyle A}$	$97.91\pm2.67^{\rm a}$	$114\pm1.32^{\rm A}$	0.001	
Waist-to-hip ratio	$0.91\pm0.00^{\rm a}$	$1.03\pm0.24^{\rm A}$	$0.99\pm0.00^{\rm a}$	$0.99\pm0.01^{\rm a}$	0.002	
Biochemical						
FBG (mg/dl)	95.15 ± 1.53^{ab}	$97.92\pm1.42^{\text{ab}}$	$169.45 \pm 13.59^{\rm A}$	158.28 ± 9.71^{aB}	0.001	
HbA1C (%)	$5.12\pm0.10^{\scriptscriptstyle A}$	$5.43\pm0.08^{\rm A}$	$7.12\pm0.31^{\rm a}$	$7.13\pm0.28^{\rm a}$	0.001	
Cortisol (µg/mL)	$12.92\pm0.91^{\rm a}$	$11.01\pm0.66^{\rm a}$	$14.21\pm0.91^{\scriptscriptstyle A}$	$15.15\pm0.91^{\scriptscriptstyle A}$	0.005	
Serum testosterone (ng/mL)	$4.47\pm0.29^{\rm A}$	$3.52\pm0.23^{\rm a}$	$3.78\pm0.26^{\rm a}$	$3.70\pm0.43^{\rm a}$	0.017	

Control; Normal weight and non-diabetic mellitus (BMI <25 kg/m²), Obese; Obese and non-diabetic mellitus (BMI \ge 30 kg/m²), Nob-DM; Diabetic mellitus non-obese (BMI \le 30 kg/m²), Ob-DM; Diabetic and obese men (BMI \ge 30 kg/m²), BMI; Body mass index, FBG; Fasting blood glucose, and HbA1C; Glycosylated hemoglobin. Capital letters vs. their corresponding lowercases (A vs. a, B vs. b, and C vs. c) indicate a significant difference (P<0.05). Data are presented as mean \pm SE.

Sperm concentration and motility and normal morphology

To clarify the effect of obesity and diabetes on the sperm parameters, sperm concentration, motility and morphology were detected (Table 2). Concerning the same period of sexual abstinence for all groups, there was statistically significant difference in sperm concentration, total motility, progressive motility, and total sperm counts, except the volume, semen pH, and sperm density between the groups.

Oxidative stress assessment

The data regarding seminal plasma concentrations of ROS, TAC, DNA fragmentation, and annexin-V binding assay are summarized in Table 3. ROS levels were significantly lower in the control group in comparison to other groups (P<0.001). Moreover, sperm DNA fragmentation was higher in diabetic and obese groups as compared with the control group (P<0.05).

As expected, TAC values were lower in Nob-DM, ob-

DM, and ob-Non-DM groups as compared with the control group. On the other hand, in the control group, the percentage of viable cells (Anx⁻ PI⁻) was higher than that of other groups, which means that the control group had significantly lower numbers of apoptotic cells (Anx⁺ PI⁺) sperm. An inverse correlation was found between sperm ROS levels and sperm motility (total or progressive). The percentage of live viable cells (Anx⁻ PI⁻) had a positive association with motility and morphology percentage (Table 4).

Hormonal assays

Serum cortisol levels were significantly different when compared between diabetic patients (Nob-DM and ob-DM) and healthy individuals (P<0.05). However, no significant difference was found between serum cortisol level between the obese and control groups.

Serum testosterone levels were significantly higher in the control group compared with other groups (P<0.05). A negative association was found between the testosterone and cortisol levels in all groups of patients (Table 4).

Table 2: Comparison of semen parameters between patients and the control groups. The numbers are adjusted in compliance with age, and BMI

Parameter					
	Control	Obese	Nob-DM	Ob-DM	P value
	n=40	n=40	n=35	n=35	
Semen volume (ml)	3.1 ± 0.4	2.8 ± 0.3	2.8 ± 0.8	2.7 ± 0.9	0.018
Semen pH	7.6 ± 0.3	7.8 ± 0.2	7.5 ± 0.1	7.5 ± 0.4	0.020
sperm concentration (10 ⁶ ml ⁻¹)	$71.40\pm4.97^{\scriptscriptstyle A}$	$47.52\pm5.03^{\text{a}}$	$47.36\pm5.15^{\rm a}$	$48.93\pm5.49^{\mathrm{a}}$	0.002
Sperm motility (%)	$74.19\pm2.41^{\scriptscriptstyle A}$	$55.96\pm3.60^{\rm a}$	$56.03\pm3.45^{\rm a}$	$54.84\pm3.83^{\mathrm{a}}$	0.007
Normal sperm morphology (%)	$28.50\pm1.81^{\scriptscriptstyle A}$	$13.57\pm1.47^{\rm a}$	$11.65\pm2.14^{\rm a}$	$16.68\pm2.09^{\mathrm{a}}$	0.000
Progressive motility (%)	$49.07\pm2.79^{\scriptscriptstyle A}$	$37.13\pm3.67^{\rm a}$	$37.02\pm3.72^{\rm a}$	$33.39\pm3.28^{\rm a}$	0.004
Total normal progressively motile sperm (n)	$73.71\pm2.70^{\scriptscriptstyle A}$	$62.23\pm3.34^{\rm a}$	$61.39\pm5.01^{\rm a}$	$56.28\pm3.69^{\mathrm{a}}$	0.006

Control; Normal weight and non-diabetic mellitus (BMI <25 kg/m²), Obese; Obese and non-diabetic mellitus (BMI \ge 30 kg/m²), Nob-DM; Diabetic mellitus non-obese (BMI \le 30 kg/m²), Ob-DM; Diabetic and obese men (BMI \ge 30 kg/m²), and BMI; Body mass index. Capital versus small letters (A with a, B with b and C with c) indicated significantly different (P<0.05). Data are presented as mean ± SE.

Table 3: Comparison of oxidative stress biomarkers and apoptosis between the patient and control groups. The numbers are adjusted in compliance with age, and BMI

Variable	Groups					
	Control	Obese	Nob-DM	Ob-DM	P value	
	n=40	n=40	n=35	n=35		
ROS (RLU/sec/×10 ⁶ sperm)	$40.85\pm0.74^{\rm a}$	$66.03\pm6.77^{\scriptscriptstyle A}$	$54.46\pm6.37^{\rm A}$	$48.52\pm3.02^{\scriptscriptstyle A}$	0.001	
TAC (nmol/µl)	$60.96\pm3.04^{\scriptscriptstyle A}$	$49.47\pm2.19^{\rm a}$	$49.69\pm2.82^{\rm a}$	$48.54\pm2.68^{\rm a}$	0.001	
SDF (%)	$26.38\pm1.63^{\rm a}$	$49.12\pm3.18^{\scriptscriptstyle A}$	$47.82\pm1.99^{\scriptscriptstyle A}$	$51.67\pm2.07^{\scriptscriptstyle A}$	0.003	
PI ⁻ and Anx ⁻ (%)	$61.17\pm1.71^{\scriptscriptstyle A}$	$41.66\pm2.86^{\rm a}$	$46.69\pm2.51^{\rm a}$	$42.23\pm3.42^{\rm a}$	0.005	
PI^{-} and Anx^{+} (%)	$12.67\pm1.49^{\rm a}$	$23.78\pm3.31^{\scriptscriptstyle A}$	$15.75\pm2.34^{\rm a}$	$26.80\pm4.13^{\scriptscriptstyle A}$	0.001	
PI^{+} and Anx^{+} (%)	$16.23\pm1.37^{\rm a}$	$24.95\pm2.34^{\scriptscriptstyle A}$	$28.53\pm2.40^{\scriptscriptstyle A}$	$26.76\pm1.83^{\scriptscriptstyle A}$	0.018	
PI ⁺ and Anx ⁻ (%)	$9.93 \pm 1.95^{\rm A}$	$10.68\pm2.68^{\rm A}$	$9.84\pm2.20^{\rm A}$	$4.12\pm0.72^{\rm a}$	0.016	

Control; Normal weight and non-diabetic mellitus (BMI <25 kg/m²), Obese; Obese and non-diabetic mellitus (BMI \ge 30 kg/m²), Nob-DM; Diabetic mellitus non-obese (BMI <30 kg/m²), Ob-DM; Diabetic and obese men (BMI \ge 30 kg/m², BMI; Body mass index, ROS; Reactive oxygen species, TAC; Total antioxidant capacity, SDF; Sperm DNA fragmentation, Anx; Annexin V-negative, Anx⁺; Annexin V-positive, PI⁺; Propidium iodide positive, and PI; Propidium iodide-negative. Capital letters versus same small letters (A with a, B with b and C with c) indicated significantly different (P<0.05). Data are presented as mean ± SE.

 Table 4: Correlations among semen quality, obesity-associated markers, hormone, and oxidative stress biomarkers in all individuals participated in the study

	ncentration (10^6 ml^{-1})	otility (%)	oerm morphology (%)	/e motility (%)						ng/mL)	ıg/mL)	U/sec/ × 106 sperms)	(Iŋ/G		ux ⁺ (%)	IX ⁺ (%)
Variable	Sperm con	Sperm mo	Normal sp	Progressiv	HbA1C	FBS	BMI	Waist	Hip	Serum T (Cortisol (µ	ROS (RL)	TAC (nmc	SDF (%)	PI ⁺ and A ₁	PI and Ar
Sperm concentration (10^6 ml ⁻¹)	1	0.58*	0.45*	0.46*	-0.16*	-0.23*	-0.20*	-0.19*	-0.18*	0.12	-0.10	-0.11	0.14	-0.38*	-0.02	0.17*
Sperm motility (%)		1	0.41*	0.86*	-0.14*	-0.24*	-0.31*	-0.30*	-0.24*	0.11	-0.07	-0.25*	0.13	-0.47*	-0.09	0.10
Normal sperm morphology (%)			1	0.33*	-0.26*	-0.31*	-0.35*	-0.38*	-0.34*	-0.11	-0.02	-0.09	0.08	-0.38*	-0.16*	-0.19*
Progressive motility (%)				1	-0.10*	-0.23*	-0.22*	-0.24*	-0.19*	-0.04	-0.11	-0.23*	0.08	-0.40*	-0.17*	-0.24*
HbA1C					1	0.71*	0.22*	0.21*	0.18*	0.16*	0.22*	-0.06	-0.15	0.32*	0.23*	0.05
FBS						1	0.17*	0.15*	0.13*	0.16*	0.37*	0.00	-0.11	0.26*	0.17*	0.03
BMI							1	0.91*	0.88*	-0.28*	-0.09	0.06	-0.19*	0.54*	0.16*	0.22*
Waist								1	0.88*	-0.21*	-0.06	0.09	-0.19*	0.53*	0.18*	0.16*
Hip									1	-0.14	0.02	-0.02	-0.21*	0.44*	0.19*	0.21*
Serum T (ng/mL)										1	0.13	-0.01	0.02	-0.17	-0.11	0.04
Cortisol (µg/mL)											1	-0.16	-0.15	0.03	0.05	-0.10
ROS (RLU/sec/×10 ⁶ sperm)												1	0.03	0.17	-0.08	0.03
TAC (nmol/µl)													1	-0.18	-0.16	0.03
SDF (%)														1	0.09	0.22*
$PI^{\scriptscriptstyle +}$ and $Anx^{\scriptscriptstyle +}$ (%)															1	-0.18*
$PI^{\scriptscriptstyle -}$ and $Anx^{\scriptscriptstyle +}$ (%)																1

HbA1C; Glycosylated hemoglobin, FBS; Fasting blood glucose, BMI; Body mass index, ROS; Reactive oxygen species, TAC; Total antioxidant capacity, SDF; Sperm DNA fragmentation, Anx⁺; Annexin V-positive, PI⁺; Propidium iodide positive, PI⁻; Propidium iodide-negative, and ^{*}; P<0.05.

Discussion

We observed elevated ROS levels in sperm of obese and diabetic men when they were compared with the control group. As visceral fat store expands, adipocytes generate increasing levels of ROS. Hyperglycemia appears to have a pivotal role in diabetic complications due to the excessive production of ROS. Additionally, the main source of endogenous ROS in semen samples of obese (20-22) and diabetic patients (23, 24) is attributed to the leukocytes, as well as the defective function of those cells in patients with obesity and diabetic. The increased amounts of ROS in obese and diabetic patients highlight

the importance of free radical agents in the development of infertility in men who are affected with the earlymentioned disorders (25, 26).

Oxidative stress is a condition where the production of ROS surpasses the antioxidant levels. Several studies have also shown that the seminal plasma TAC concentrations were lower in sub-fertile and infertile men than the healthy male subjects (27-29). In our study, the concentration of blood plasma antioxidant was higher in obese and diabetic men in comparison with other groups.

ROS play a crucial role in several reproductive stages

in capacitation, acrosome reaction, fertilization, and normal development and maturation of spermatozoa (30). Heightened levels of ROS have been associated with impaired sperm motility, sperm concentration, and sperm morphology (31, 32),

Apoptosis is an autonomous programmed cell death process that is stimulated under specific conditions. In the present study, following an increase in ROS levels in obese and diabetics patients, the percentage of live normal spermatozoa (Anx⁻, PI⁻) was diminished.

Elevated levels of ROS can cause transitory loss of the mitochondrial membrane potential and form mitochondrial pores in the inner membrane by which the egress of cytochrome c is capable of activating the complex of apoptosome followed by the activation of executioner caspases, exposure of PS on the external leaflet of the plasma membrane, and ultimately cell death (33).

Also, it seems that damage to the DNA contents of sperm is mainly caused by mitochondrial ROS generation that can stem from damaged spermatozoa (5, 34). The identification of DNA damage is an important factor in the evaluation of semen quality and considered a useful marker in the diagnosis of male infertility (35).

Our results show that acute exposure to ROS can induce DNA damage in the sperm of obese, Nob-DM, and Obese-DM patients. Due to the possible effects of oxidative stress, sperm apoptosis, and DNA fragmentation on semen parameters, we investigated the correlations of semen parameters with oxidative stress status. We found that stress, sperm apoptosis, and DNA fragmentation were linked with sperm parameter (except sperm motility). A negative relationship was shown between ROS and sperm parameters (36-38).

There are relatively few studies that assessed the association between ROS generation and stress-related hormones in fertility problems. According to Darbandi et al. (39), following the generation of ROS, the HPA axis becomes activated and releases cortisol in humans in response to stress In the present study, a higher cortisol level was identified in diabetic men (Nob-DM and ob-DM) compared with the control group, but there was no any association between cortisol and sperm parameters in all studied groups. The statistically significant correlation between cortisol and testosterone is the most important finding, supporting the fact that the inhibition of testosterone synthesis is a reflection of cortisol elevation.

It seems that cortisol, through the cross-talk between the HPG and HPA axes, as well as the reduction of testosterone levels in response to excessive amounts of ROS, might have roles in alteration of sperm parameters (40).

The present study is the first comprehensive observation of the categorization of metabolic diseases and oxidative stress assessments. Moreover, in this study, one of the limitations was the lack of our knowledge about the patient's lifestyle (especially for physical activity). Although we tried to age-matched our studied groups, it was not possible even with adjusting the age between experimental groups.

Conclusion

Regarding the obtained results, increased levels of ROS were detected in sperm of obese and diabetics men, and therefore all men with diabetes and obesity should undergo screening for ROS because high levels of ROS impaired mitochondrial function and ultimately lead to DNA damage and impairment HPG/HPA axis. ROS could be considered the economical and simple technique predictive tool that can reflect an individual's susceptibility to developing diabetes- and obesity-induced complications such as infertility in men.

Acknowledgements

We thank Dr. Zahednasab for the proof-reading and edition of the manuscript. This study was financially supported by Royan Institute. The authors declare no conflict of interests.

Authors' Contributions

A.Sh.; Conception and design of the study. V.B., A.K.; Contributed to conception and design and manuscript drafting and revising. K.A.; Conducted experiments and collected data. Z.Z.; Analyzed and interpreted the data. A.N.N.; Contributed to conception and design. Sh.A.; Contributed to the study design, data collection, preparation of the initial manuscript, revision and finalization of the manuscript. All authors read and approved the final manuscript.

References

- 1. Omolaoye TS, Skosana BT, du Plessis SS. Diabetes mellitus- induction: Effect of different streptozotocin doses on male reproductive parameters. Acta Histochem. 2018; 120(2): 103-109.
- Jensen TK, Andersson AM, Jorgensen N, Andersen AG, Carlsen E, Petersen JH, et al. Body mass index in relation to semen quality and reproductive hormones among 1,558 Danish men. Fertil Steril. 2004; 82(4): 863-870.
- 3. James PT, Rigby N, Leach R; International Obesity Task Force. The obesity epidemic, metabolic syndrome and future prevention strategies. Eur J Cardiovasc Prev Rehabil. 2004; 11(1): 3-8.
- Chen H, Zhao HX, Huang XF, Chen GW, Yang ZX, Sun WJ, et al. Does high load of oxidants in human semen contribute to male factor infertility? Antioxid Redox Signal. 2012; 16(8): 754-759.
- Aitken RJ, De Iuliis GN, Finnie JM, Hedges A, McLachlan RI. Analysis of the relationships between oxidative stress, DNA damage and sperm vitality in a patient population: development of diagnostic criteria. Hum Reprod. 2010; 25(10): 2415-2426.
- Kumar N, Singh AK. Reactive Oxygen Species in Seminal Plasma as a Cause of Male Infertility. J Gynecol Obstet Hum Reprod. 2018; 47(10): 565-572.
- Agarwal A, Prabakaran S, Allamaneni S. What an andrologist/ urologist should know about free radicals and why. Urology. 2006; 67(1): 2-8.
- Aitken RJ, Smith TB, Jobling MS, Baker MA, De Iuliis GN. Oxidative stress and male reproductive health. Asian J Androl. 2014; 16(1): 31-38.
- Gavriliouk D, Aitken RJ. Damage to sperm DNA mediated by reactive oxygen species: its impact on human reproduction and the health trajectory of offspring. Adv Exp Med Biol. 2015; 868: 23-47.
- Aitken RJ, Gibb Z, Baker MA, Drevet J, Gharagozloo P. Causes and consequences of oxidative stress in spermatozoa. Reprod Fertil Dev. 2016; 28(1-2): 1-10.

- Du Plessis SS, Cabler S, McAlister DA, Sabanegh E, Agarwal A. The effect of obesity on sperm disorders and male infertility. Nat Rev Urol. 2010; 7(3): 153-161.
- Costantini D, Marasco V, Moller AP. A meta-analysis of glucocorticoids as modulators of oxidative stress in vertebrates. J Comp Physiol B. 2011; 181(4): 447-456.
- Costantini D, Møller AP. Does immune response cause oxidative stress in birds? A meta-analysis. Comp Biochem Physiol A Mol Integr Physiol. 2009; 153(3): 339-344.
- Abraham SB, Rubino D, Sinaii N, Ramsey S, Nieman LK. Cortisol, obesity, and the metabolic syndrome: a cross-sectional study of obese subjects and review of the literature. Obesity (Silver Spring). 2013; 21(1): E105-E117.
- Cooper TG, Noonan E, von Eckardstein S, Auger J, Baker HW, Behre HM, et al. World Health Organization reference values for human semen characteristics. Hum Reprod Update. 2010; 16(3): 231-245.
- Abbasihormozi S, Kouhkan A, Alizadeh AR, Shahverdi AH, Nasr-Esfahani MH, Sadighi Gilani MA, et al. Association of vitamin D status with semen quality and reproductive hormones in Iranian subfertile men. Andrology. 2017; 5(1): 113-118.
- Ahmadi MH, Mirsalehian A, Sadighi Gilani MA, Bahador A, Talebi M. Asymptomatic infection with Mycoplasma hominis negatively affects semen parameters and leads to male infertility as confirmed by improved semen parameters after antibiotic treatment. Urology. 2017; 100: 97-102.
- Tavalaee M, Deemeh MR, Arbabian M, Nasr-Esfahani MH. Density gradient centrifugation before or after magnetic-activated cell sorting: which technique is more useful for clinical sperm selection? J Assist Reprod Genet. 2012; 29(1): 31-38.
- Evenson DP. Sperm chromatin structure assay (SCSA®). Methods Mol Biol. 2013; 927: 147-164.
- Carvalheira JB, Qiu Y, Chawla A. Blood spotlight on leukocytes and obesity. Blood. 2013; 122(19): 3263-3267.
- Minervino D, Gumiero D, Nicolazzi MA, Carnicelli A, Fuorlo M, Guidone C, et al. Leukocyte activation in obese patients: effect of bariatric surgery. Medicine (Baltimore). 2015; 94(40): e1382.
- Ryder E, Diez-Ewald M, Mosquera J, Fernandez E, Pedreanez A, Vargas R, et al. Association of obesity with leukocyte count in obese individuals without metabolic syndrome. Diabetes Metab Syndr. 2014; 8(4): 197-204.
- Moradi S, Kerman SR, Rohani F, Salari F. Association between diabetes complications and leukocyte counts in Iranian patients. J Inflamm Res. 2012; 5: 7-11.
- Menart-Houtermans B, Rutter R, Nowotny B, Rosenbauer J, Koliaki C, Kahl S, et al. Leukocyte profiles differ between type 1 and type 2 diabetes and are associated with metabolic phenotypes: results from the German Diabetes Study (GDS). Diabetes Care. 2014; 37(8): 2326-2333.
- Hamada AJ, Montgomery B, Agarwal A. Male infertility: a critical review of pharmacologic management. Expert Opin Pharmacother.

2012; 13(17): 2511-2531.

- Tremellen K. Oxidative stress and male infertility--a clinical perspective. Hum Reprod Update. 2008; 14(3): 243-258.
- Pasqualotto FF, Sharma RK, Pasqualotto EB, Agarwal A. Poor semen quality and ROS-TAC scores in patients with idiopathic infertility. Urol Int. 2008; 81(3): 263-270.
- Siciliano L, Tarantino P, Longobardi F, Rago V, De Stefano C, Carpino A. Impaired seminal antioxidant capacity in human semen with hyperviscosity or oligoasthenozoospermia. J Androl. 2001; 22(5): 798-803.
- Hosseinzadeh Colagar A, Karimi F, Jorsaraei SG. Correlation of sperm parameters with semen lipid peroxidation and total antioxidants levels in astheno- and oligoasheno- teratospermic men. Iran Red Crescent Med J. 2013; 15(9): 780-785.
- Mahfouz R, Sharma R, Thiyagarajan A, Kale V, Gupta S, Sabanegh E, et al. Semen characteristics and sperm DNA fragmentation in infertile men with low and high levels of seminal reactive oxygen species. Fertil Steril. 2010; 94(6): 2141-2146.
- Sawyer DE, Mercer BG, Wiklendt AM, Aitken RJ. Quantitative analysis of gene-specific DNA damage in human spermatozoa. Mutat Res. 2003; 529(1-2): 21-34.
- Agarwal A, Saleh RA. Role of oxidants in male infertility: rationale, significance, and treatment. Urol Clin North Am. 2002; 29(4): 817-827.
- Padron OF, Brackett NL, Sharma RK, Lynne CM, Thomas AJ Jr, Agarwal A. Seminal reactive oxygen species and sperm motility and morphology in men with spinal cord injury. Fertil Steril. 1997; 67(6): 1115-1120.
- Madungwe NB, Zilberstein NF, Feng Y, Bopassa JC. Critical role of mitochondrial ROS is dependent on their site of production on the electron transport chain in ischemic heart. Am J Cardiovasc Dis. 2016; 6(3): 93-108.
- Bui AD, Sharma R, Henkel R, Agarwal A. Reactive oxygen species impact on sperm DNA and its role in male infertility. Andrologia. 2018; 50(8): e13012.
- Agarwal A, Durairajanayagam D, Halabi J, Peng J, Vazquez-Levin M. Proteomics, oxidative stress and male infertility. Reprod Biomed Online. 2014; 29(1): 32-58.
- Vatannejad A, Tavilani H, Sadeghi MR, Amanpour S, Shapourizadeh S, Doosti M. Evaluation of ROS-TAC score and DNA damage in fertile normozoospermic and infertile Asthenozoospermic Males. Urol J. 2017; 14(1): 2973-2978.
- Agarwal A, Saleh RA, Bedaiwy MA. Role of reactive oxygen species in the pathophysiology of human reproduction. Fertil Steril. 2003; 79(4): 829-843.
- Darbandi M, Darbandi s, Aggarwal A, Sengupta P, Durairajanayagam D, Henkel R, et al. Reactive oxygen species and male reproductive hormones. Reprod Biol Endocrinol. 2018; 16(1): 87.
- 40. Jia YF, Feng Q, Ge ZY, Guo Y, Zhou F, Zhang KS, et al. Obesity impairs male fertility through long-term effects on spermatogenesis. BMC Urol. 2018; 18(1): 42.

Reduction of truncated Kit Expression in Men with Abnormal Semen Parameters, Globozoospermia and History of Low or Fertilization Failure

Somayeh Haghighat, M.Sc.^{1, 2#}, Marziyeh Tavalaee, Ph.D.^{2#}, Zahra Zakeri, Ph.D.³, Mahdi Noureddini, Ph.D.^{1*},

Abdolhossein Shahverdi, Ph.D.⁴, Mohammad Hossein Nasr Esfahani, Ph.D.^{2, 5*}

1. Physiology Research Center, Kashan University of Medical Sciences, Kashan, Iran

2. Department of Reproductive Biotechnology, Reproductive Biomedicine Research Center, Royan Institute for Biotechnology, ACECR, Isfahan, Iran

3. Department of Biology, Queens College and Graduate Center of the City University of New York, Flushing, NY, USA

4. Department of Embryology, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Medicine, ACECR,

Tehran, Iran

5. Isfahan Fertility and Infertility Center, Isfahan, Iran

#The first two authors equally contributed to this work.

*Corresponding Addresses: P.O.Box: 87155/111, Physiology Research Center, Kashan University of Medical Sciences, Kashan, Iran P.O.Box: 8165131378, Department of Reproductive Biotechnology, Reproductive Biomedicine Research Center, Royan Institute for Biotechnology, ACECR, Isfahan, Iran

Emails: mnoureddini@yahoo.com, mh.nasr-esfahani@royaninstitute.org

Received: 19/June/2018, Accepted: 22/October/2018 Abstract

Objective: Phospholipase C zeta 1 (PLC ζ) is one of the main sperm factor involved in oocyte activation and other factors may assist this factor to induce successful fertilization. Microinjection of recombinant *tr-kit*, a truncated form of c-kit receptor, into metaphase II-arrested mouse oocytes initiate egg activation. Considering the potential roles of tr-KIT during spermiogenesis and fertilization, we aimed to assess expression of tr-KIT in sperm of men with normal and abnormal parameters and also in infertile men with previous failed fertilization and globozoospermia.

Materials and Methods: This experimental study was conducted from September 2015 to July 2016 on 30 normozoospermic and 20 abnormozoospermic samples for experiment one, and also was carried out on 10 globozoospermic men, 10 men with a history low or failed fertilization and 13 fertile men for experiment two. Semen parameters and sperm DNA fragmentation were assessed according to WHO protocol, and TUNEL assay. Sperm tr-KIT was evaluated by flow cytometry, immunostaining and western blot.

Results: The results show that tr-KIT mainly was detected in post-acrosomal, equatorial and tail regions. Percentage of tr-KIT-positive spermatozoa in abnormozoospermic men was significantly lower than normozoospermic men. Also significant correlations were observed between sperm tr-KIT with sperm count (r=0.8, P<0.001), motility (r=0.31, P=0.03) and abnormal morphology (r=-0.6, P<0.001). Expression of tr-KIT protein was significantly lower in infertile men with low/ failed fertilization and globozoospermia compared to fertile men. The significant correlations were observed between sperm tr-KIT protein with fertilization rate (r=-0.46, P=0.04). In addition, significant correlations were observed between sperm DNA fragmentation with fertilization rate (r=-0.56, P=0.019) and tr-KIT protein (r=-0.38, P=0.04).

Conclusion: tr-KIT may play a direct or indirect role in fertilization. Therefore, to increase our insight regarding the role of tr-KIT in fertilization further research is warranted.

Keywords: DNA Fragmentation, Fertilization, Globozoospermia, Male Infertility

Cell Journal (Yakhteh), Vol 21, No 3, October-December (Autumn) 2019, Pages: 314-321 ____

Citation: Haghighat S, Tavalaee M, Zakeri Z, Noureddini M, Shahverdi AH, Nasr Esfahani MH. Reduction of truncated kit expression in men with abnormal semen parameters, globozoospermia and history of low or fertilization failure. Cell J. 2019; 21(3): 314-321. doi: 10.22074/cellj.2019.6112.

Introduction

Intra-cytoplasmic sperm injection (ICSI) technique has been applied increasingly to treat sperm-related infertility. During this technique, natural sperm selection barriers present in female reproductive tract and also initial physiological process of fertilization such as capacitation and acrosome reaction are bypassed. Therefore, it allows couples with little hope of achieving successful pregnancy to acquire fruitful fertilization and pregnancy (1-4). Despite this potential, low or complete fertilization failure still occurs in a considerable number of ICSI cases. This phenomenon is mainly related to inability of sperm to induce oocyte activation (5, 6). In this regard, Swain and pool (6) showed that over 50% etiology of failed fertilization post *in vitro* fertilization (IVF) is related to failed oocyte activation and so far several sperm factors are described to be involved in oocyte activation, including: testis-specific phospholipase C zeta 1 (PLC ζ), postacrosomal sheath WW domain-binding protein (WBP2NL or PAWP) and truncated *c-kit* gene product (*tr-KIT*) (7-12). These factors, commonly termed "spermborne oocyte activation factors (SOAFs)", are released into ooplasm upon fusion of sperm with oocyte and lead to intracellular calcium oscillation (13).

A large body of consistent and reproducible evidence suggests that PLC ζ is the main factor that instigate Ca²⁺ release from intracellular stores (11, 13-17) and other factors may assist this protein in this process. Evidence

may suggest that WBP2NL/PAWP may complement action of PLC ζ by activating PLC γ noncanonically (7, 8, 18-21). Possibly, PAWP acquires this action via Yes-Associated Protein (YAP) which has an SH3 binding motif and this motif interacts with an SH3 domain in PLC γ . Therefore, Ca²⁺ release from intracellular stores via the PIP, vesicles is initiated. Even though this signaling pathway has been envisaged for PAWP, but further verifications are required (10, 22). Similarly, shortened cytoplasmic product of c-KIT, called tr-KIT activate Fyn (a Src-like kinase) and subsequently SH3 binding motif of this kinase interacts with the SH3 domain in PLC γ to induce Ca²⁺ release from intracellular stores (22, 23). In this regard, Rossi et al. (22) suggested "microinjection of *tr-kit* into mouse eggs causes their parthenogenetic activation (12, 23). Thus, tr-KIT is a candidate as an assistant sperm factor that might play a role in the final function of the gametes, fertilization." However, further verification of these pathway remains to be explored.

C-KIT is important to gametogenesis in controlling primordial germ cell survival and also spermatogonial proliferation. It is highly expressed in spermatogonia and spermatocytes; and its expression declines in round and elongated spermatids; then it is completely absent from mature spermatozoa (22, 24). In contrast, expression of tr-KIT is very low in spermatogonia and spermatocytes, while its expression considerably increases in late spermiogenesis especially in mature spermatozoa (25). tr-KIT is present in the equatorial segment, midpiece and sub-acrosomal regions of the human sperm head and its presence has been associated with sperm motility, acrosome reaction and oocyte activation in mice (23, 26). Considering the potential roles of tr-KIT during spermiogenesis and fertilization, we aimed to evaluate expression of tr-KIT in semen samples of men with normal and abnormal parameters and also in semen samples of infertile men with previous failed fertilization and globozoospermia.

Materials and Methods

Ethical approval

This experimental study has been approved by the Ethics Committee of Royan Institute (94000127). Written consent was obtained from all patients and their partners included in this study.

Experiment one: assessment of tr-KIT in individuals with normal and abnormal semen parameters

Study population and semen samples analysis

Ejaculated semen was obtained from 50 men who were referred for semen analysis to the Andrology Unit of Isfahan Fertility and Infertility Center (IFIC). Semen parameters were assessed according to WHO (27) protocol and semen samples were considered as normal or abnormal according to the WHO-2010 criteria. Individuals with sperm concentration ≥ 15 million per ml, total sperm count of > 35 million per ejaculate, percentage total motility higher than 40% and/or percentage abnormal morphology of lower than 96% were considered as "normozoospermic" or "normal parameters" group. Based on this categorization 30 individuals were selected for normozoospermia group and 20 individuals with at least two abnormal sperm parameters were included in "abnormozoospermia" or abnormal parameters" group. Semen samples with greater than one million WBC or other cell types were also excluded from our study. The remaining semen samples were used for assessment of tr-KIT by flow cytometry.

Verification of expression of *tr-KIT* and *c-KIT*

In order to assess expression of *tr-KIT* and *c-KIT* in sperm, one pair of primer for *tr-KIT* (phosphotransferase domain) and one pair of primer for *c-KIT* (ligand-binding domain), were designed. Then, expression of *tr-KIT* and *c-KIT* were assessed in one testicular biopsy from obstructive azoospermia undergoing ICSI, washed, and processed semen samples from normozoospermic individuals (n=5) by real time polymerase chain reaction (PCR) (Fig.1). In addition, western blot analyses were carried on sperm from fertile and infertile men in experimental two for detection of tr-KIT and *c*-KIT bands in sperm by an anti-human primary antibody (Santa Cruz, USA).



Fig.1: Assessment of *c-KIT* and *tr-KIT* transcripts in a testicular tissue, fresh semen (n=5), and DGC processed semen (n=5) samples. DGC; Density gradient centrifugation, and #; Shows a significant difference between fresh semen and DGC processed semen (n=5) samples at P<0.05.

Assessment of tr-KIT by flow cytometry

Briefly, semen samples from both groups were washed twice in phosphate-buffered saline (PBS) and fixed in cold acetone. Then, sperm pellets were washed twice with PBS for 5 minutes at 3000 rpm and incubated with bovine serum albumin (BSA, Sigma-Aldrich, USA) 5%+normal goat serum (NGS, Chemicon, Germany) 10% for 2 hours to block non-specific binding sites. Next, the affinitypurified anti-human primary antibody (Santa Cruz, USA) in PBS containing 1% bovine serum albumin (BSA) was applied overnight at 4°C [this antibody detect both tr-KIT and c-KIT bands at 150 and 30 kDa, according to previous published paper by Muciaccia et al. (26), respectively. The result of this study and previous studies (22) show that unlike tr-KIT, expression of c-KIT protein is not observable in sperm. Therefore, we used C-19 antibody for assessment of tr-KIT by flow cytometry in individuals with normal and abnormal semen parameters]. Subsequently, samples were washed with PBS and incubated with goat anti-rabbit IgG secondary antibody complexed with FITC (Sigma, USA) for 1 hour at 37°C. Ultimately, samples were washed with PBS and stained with propidium iodide (1 µg/ml, Sigma-Aldrich, USA). The percentage of tr-KIT-positive spermatozoa, propidium iodide positive sperm- population was assessed by a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) by means of an argon laser with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. The analysis was carried out with subtraction of the fluorescence of control sample from test sample. For each assay, a minimum of 10,000 sperm were examined and the data were analyzed using BD CellQuest Pro software. Assessment of tr-KIT was performed according to modified protocols by Muciaccia et al. (26). Similar procedure was used for determine of tr-KIT localization by fluorescence microscope and, for each sample negative control were prepared without primary antibody.

Experiment two: assessment of tr-KIT expression and DNA fragmentation in fertile and infertile men with globozoospermia and failed fertilization

Study population and semen samples analysis

Freshly ejaculated semen was obtained from 33 fertile and infertile men attending the Andrology Unit of IFIC. Infertile men were divided two groups; globozoospermia [(100% round-headed without acrosome) (n=10)] and individuals with a history failed or low fertilization (n=10). Individuals with total fertilization failure were considered as "failed fertilization" and those with fertilization rate of lower than 25% were considered as "low fertilization". Thirteen fertile individuals who were referred for embryo donation or family balancing were considered as fertile or control group. For globozoospermia, protein was obtained from Royan protein bank (September 2013 to July 2016). For this study, individuals with failed and low fertilization were asked to voluntarily produce a second semen sample, within 7 days following ICSI. The remaining semen sample from individuals referred for embryo donation or family balancing was also used. Fertilization was assessed by the presence of pronuclei around 16-18 hour post-ICSI. The fertilization rate was calculated from the ratio of fertilized oocvtes to the total number of survived injected metaphase II oocytes, multiplied by 100.

After liquefaction of semen at room temperature, each sample was divided into two parts. The first portion was used immediately for semen analysis and assessment of sperm DNA fragmentation using TUNEL assay. For fertile individuals a portion of semen sample was initially used for density gradient centrifugation (DGC) processing for ICSI/IVF and the remaining portion was used for semen analysis and TUNEL assay. All the studied samples had somatic cell count of less than one million per ml. The second portion was used for western blot after washing with PBS and centrifugation at 220 g for 10 minutes. The cell pellet was used for protein extraction.

Assessment of sperm DNA fragmentation

Assessment of DNA fragmentation in sperm sample were carried out by a detection kit (Apoptosis Detection System Fluorescein, Promega, Germany). Briefly, sperm concentration was assessed and samples were washed in PBS. Then, 20-40 µl of washed sperm was smeared onto slides and fixed in 4% paraformaldehyde for 30 minutes at room temperature. After washing the slides, the sperm were treated with 0.2% Triton X-100 (Merck, Germany) for 5 minutes and washed twice in PBS again. The samples were covered with cover slips. For each sample, 200 randomly spermatozoa were counted using an Olympus fluorescence microscope (BX51, Japan) with the appropriate filters (460-470 nm) at ×100 magnification. The percentage of green fluorescing sperm (TUNEL positive) as fragmented DNA in sperm was reported (28).

RNA isolation and quantitative real-time polymerase chain reaction

For RNA extraction and cDNA synthesis, we used Aghajanpour et al. (29) protocol. Briefly, semen samples were washed with PBS, and total RNA was extracted using Trizol (Sigma-Aldrich, USA) for both fertile and infertile samples. For removing contamination of genomic DNA, samples were treated with DNaseI (Fermentas, USA). 1 µg of total RNA were used for cDNA synthesis by random hexamer primer and the RevertAid ™H Minus First Strand cDNA. For Realtime PCR, we used a Step One Plus thermal cycler [Applied Biosystems (ABi)], and this method was carried out according to the manufacturer's protocol (TaKaRa, Ohtsu, Japan). The PCR mixture contained 3 pmol/µl of each primer, 10 µl SYBR premix Ex Taq II (TaKaRa, Ohtsu, Japan), and 25 ng cDNA adjusted to a final volume of 20 µl using dH₂O for each reaction. All reactions were carried out in triplicate. Real-time specific primer pairs were designed by the Beacon Designer 7.5. The primers used were previously designed as:

tr-KIT-

F: 5'-CAGCCAGAAATATCCTCCTTACT-3' (Exon 17) R: 5'-GCCATCCACTTCACAGGTAG-3' (Exon 18)

GAPDH-

F: 5'-CCACTCCTCCACCTTTGACG-3' R: 5'-CCACCACCCTGTTGCTGTAG-3'

c-KIT-

F: 5'-GCGAGAGCTGGAACGTGGAC-3' (Exon 1)

R: 5'-CTGGATGGATGGATGGATGGAGAC-3' (Exon 2)

The real time PCR protocol was carried out according

Tavalaee and NasrEsfahani (30). For presentation of data, the CT of target mRNA was normalized by CT of the reference gene (*GAPDH*). The data was expressed as Δ CT (CT of target gene-CT of *GAPDH* gene). A sample with lower Δ CT indicate higher concentration of target mRNA and vice versa.

Western blot technique

Expression of tr-KIT at protein level was assessed by western blot technique in 13 fertile, 10 infertile men with failed fertilization and, 10 globozoospermic men. Briefly, semen samples were washed with PBS, and extraction of protein was carried using TRI Reagent (Sigma-Aldrich, USA). Then, protein concentration was evaluated by Bradford assay (Bio-Rad, USA). 40 µg of protein for each sample were subjected to 12% sodium dodecyl sulfatepolyacrylamide gel electrophoresis (PAGE), and then transferred to polyvinylidene fluoride [(PVDF membrane (BioRad, USA)]. For GAPDH and tr-KIT, membranes were blocked with 10% skim milk at 4°C overnight and, 5% skim milk at 4°C for 1 hour, respectively. Then, membranes were incubated with primary antibodies [GAPDH for 90 minutes, and tr-KIT for overnight at room temperature, and 4°C, respectively]. Then, membranes were washed and incubated with secondary antibodies (goat anti-rabbit IgG-HRP and Peroxidase-Conjugated Goat Anti-Mouse Immunoglobulins) for 1 hour (30). Densitometric analysis of the bands was performed by Quantity One Software v 4.6.9 (Bio-Rad, Germany). The data was normalized as mean intensity of the infertile's band/mean intensity of fertile bands, and results were expressed as mean relative intensity.

Data analysis

In this study, we used Microsoft Excel and SPSS (Version 17, Chicago, IL, USA) for data analyses. Equality of variances, and normal distribution were analyzed using Levene's test and Shapiro-Wilk, respectively. Comparison of study variations between two groups were determined with independent-samples t test while between three groups were analyzed with one-way analysis of variance (ANOVA). In addition, pearson analysis was used to assess the correlations between difference parameters. Data were expressed as mean \pm standard error of mean (SEM). A P<0.05 was statistically significant.

Results

Verification of expression of tr-KIT and *c-KIT* transcripts

Real time PCR results revealed that both *c-KIT* and *tr-KIT* transcripts are expressed by testicular tissue and sperm (Fig.1). For detection of tr-KIT and c-KIT proteins in sperm, we used C-19 antibody, according to previous published paper by Muciaccia et al. (26). The result of this study (please see experiment two) and previous studies (22) show that unlike tr-KIT, expression of c-KIT protein is not observable in sperm. Therefore, we used C-19 antibody for assessment of tr-KIT by flow cytometry in individuals with normal and abnormal semen parameters.

Experiment one: assessment of tr-KIT in men with normal parameters and abnormal parameters

Following semen analyses of couples referred to IFIC, samples were considered as abnormal (n=20) or normal (n=30) based on WHO criteria. Mean values for sperm concentration (24.15 \pm 4.76 vs. 73.84 \pm 5.48), total sperm count (100.58 \pm 23.93 vs. 341.16 \pm 31.67), and percentage sperm motility (36.47 \pm 3.78 vs. 62.16 \pm 2.19) were compared between the two groups and were significantly lower in men with abnormal semen parameters. In addition, percentage of abnormal sperm morphology (98.47 \pm 0.20 vs. 95.53 \pm 0.23) was significantly higher in men with abnormal semen parameters.

We assessed percentage of tr-KIT positive-spermatozoa by flow cytometry. Figure 2 shows that mean percentage of tr-KIT positive-spermatozoa was lower in men with abnormal semen parameters compared to men with normal semen parameters (Fig.2C, which quantifies Fig.2A, B). Localization of tr-KIT in sperm was evaluated by immunostaining method and we show that tr-KIT was mainly localized in the equatorial region of spermatozoa head and tail (Fig.2D), while tr-KIT was not detectable in these regions of spermatozoa in negative control. In addition, we observed significant correlations between percentage of tr-KIT positive-spermatozoa in the total population with sperm count (r=0.792, P<0.001), percentage sperm motility (r=0.316, P=0.034) and abnormal morphology (r=-0.595, P<0.001, Fig.3).

Experiment two: assessment of sperm parameters, expression of sperm tr-KIT, and DNA fragmentation in fertile individuals, men with failed fertilization, and globozoospermia

Assessment of sperm parameters between fertile and infertile men

The mean values for female and male age were 34.4 \pm 9.6 and 38.75 \pm 5.37 in the fertile group; 26.4 \pm 5.59 and 30.00 ± 3.93 in globozoospermia group and, $33.78 \pm$ 2.16 and 38.45 ± 3.84 in couples with failed fertilization, respectively. In addition, sperm parameters were compared between groups. The mean values of sperm concentration were 73.76 ± 5.81 , 37.89 ± 9.34 and 30.85 ± 13.62 in fertile, infertile men with failed fertilization, and globozoospermia groups, respectively. Means of sperm concentration were significantly reduced in men with failed fertilization and globozoospermia compared to fertile individuals (P<0.001). Furthermore, means percentages sperm motility were 61.53 $\pm 2.07, 36.67 \pm 6.45, \text{ and } 22.16 \pm 9.29, \text{ in the fertile, infertile}$ men with failed fertilization, and globozoospermia groups, respectively. Similar to sperm concentration, means of percentage of sperm motility were also significantly lower in the both infertile groups compared to the fertile group. In addition, percentage of sperm abnormal morphology was significantly higher in the infertile men with globozoospermia (100%) compared to fertile (95.3 ± 0.32) and infertile men with failed fertilization (96.34 \pm 0.89) groups.



Fig.2: The results of flow cytometry and immunofluorescence staining of tr-KIT in sperm. **A.** Flow cytometric dot plot of tr-KIT in a man with normal, **B.** Abnormal semen parameters, **C.** Comparison of mean percentage of tr-KIT positive-spermatozoa between men with normal and abnormal semen parameters, and **D.** Localization of tr-KIT in sperm was evaluated by immunostaining method. #; Shows a significant difference between two groups at P<0.05.



Fig.3: Correlation between percentage of tr-KIT positive-spermatozoa with sperm count (r=0.792, P<0.001), percentage of sperm motility (r=0.316, P=0.034), and sperm abnormal morphology (r=-0.595, P<0.001, n=50).

Comparison of relative expression of sperm tr-KIT protein between fertile and infertile men

We also assessed relative expression of sperm tr-KIT protein by western blot technique in fertile, infertile men with failed fertilization and globozoospermic men. As shown in Figure 4, the band intensity of tr-KIT protein was low in infertile men with failed fertilization and globozoospermia compared to fertile individuals. Considering tr-KIT is a shortened protein produced by alternative splicing of *c-kit*, we used an antibody that able to detect both c-KIT and tr-KIT

protein at 150 and 30 kDa bands. Our result was similar to Rossi et al. (22), and we did not observe any band at 150 kDa band in sperm, while tr-KIT was detectable. We compared mean relative expression of tr-KIT protein among these groups (Fig.4). The mean of tr-KIT protein was significantly lower in infertile men with failed fertilization (0.17 ± 0.03) and globozoospermic men (0.26 ± 0.12) compared to fertile (1.7 ± 0.5) men (P<0.05). In addition, we observed a significant correlation between fertilization rate with relative expression of tr-KIT protein (r=0.46, P=0.04).



Fig.4: The results of western blots technique. **A.** Western blots of sperm tr-KIT protein from two fertile men. F; Fertile, FF; Two infertile men with failed fertilization, and G; Two infertile men with globozoospermia and **B.** Comparison of relative expression of tr-KIT protein between fertile men and both infertile groups. Arrows indicate bands of 150, 30 and 38 kDa for c-KIT, tr-KIT and GAPDH, respectively. #; Shows significant difference between fertile men and both infertile groups at P<0.05.

Comparison of sperm DNA fragmentation between fertile and infertile men

In this study, sperm DNA fragmentation was assessed by TUNEL assay in fertile and infertile men. Mean percentage of sperm DNA fragmentation was significantly higher in infertile men with failed fertilization (28.18 \pm 6.01) and globozoospermic men (23.6 \pm 5.67) compared to fertile (5.24 \pm 1.23) groups (P<0.05). In addition, we observed negative significant correlations between percentage of DNA fragmentation with fertilization rate (r=-0.45, P=0.01), and tr-KIT protein (r=-0.38, P=0.04).

Discussion

One of the cornerstones of development is ability of sperm to induce "oocyte activation". This event can initiate a series of physiological phenomena and metabolic reactions in oocyte such as release of calcium from intracellular stores, cortical granule exocytosis, block to polyspermy, and resumption of the meiotic cell cycle (15, 16). In addition to PLC ζ as main factor involved in oocyte activation, several other sperm factors suggested that may assist PLC ζ in this phenomenon (11, 9, 13). In this regard, several lines of evidence suggested low expression or absence of sperm factors involved in oocyte activation such as PLCζ and PAWP in men with low or failed fertilization post ICSI or globozoospermia (8, 29-35). Though, results of studies regarding the role of PAWP on fertilization and early embryonic development are still controversial. In this regard, Escoffier et al. (36) demonstrated that PLCζ alone is sufficient to induce oocyte activation. Among sperm factors, tr-KIT need to receive more attention in the field of male infertility.

In the mice model, previous studies showed that tr-KIT plays an important role in egg resumption from meiosis II at fertilization and zygotic development (25). In the light of these considerations, we decided to assess tr-KIT in human sperm. Our results clearly showed that both *c*-*KIT* and *tr-KIT* transcripts are present in testicular tissue and washed semen samples but western blot analysis revealed that only tr-KIT is present in sperm. Therefore,

we assessed the percentage of tr-KIT positive sperm by flow cytometry in washed semen from individuals with normal and abnormal semen parameters. Our results showed that percentage of tr-KIT-positive spermatozoa was significantly lower in sperm of men with abnormal semen parameters compared to men with normal semen parameters. Also, significant correlations were observed between percent of tr-KIT-positive spermatozoa with sperm concentration, motility, and morphology. In this regard, Muciaccia et al. (26) also observed significant correlations between percentage of sperm tr-KIT with sperm motility and morphology, but not sperm count. The difference between current study with Muciaccia et al. (26), was due to sample sizes, type of selection of patient, and used technique. Considering expression of tr-KIT is restricted to spermiogenesis phase (25), therefore defects in spermatogenesis may be lead to misregulation of expression of this protein and subsequently cause poor semen quality. In line with this concept, other studies showed that misregulation of testis-specific genes could affect spermatogenesis and thereby semen quality (29, 37, 38). Therefore, assessment of sperm tr-KIT could be considered as an additional parameter along with classic semen analysis for evaluation of semen quality.

In addition, immunostaining results show that tr-KIT is localized in the post-acrosomal and equatorial regions. This observation is in of keeping with a previous study that stated "tr-kit is not present in soluble portion of sperm, but it is found mostly in the Triton-X100 insoluble material" by western blot analysis (39). Despite this claim, we also observed tr-KIT on sperm tail region. Considering that the percentage of tr-KIT-positive spermatozoa correlated significantly with sperm motility, we explain that presence of tr-KIT on sperm tail may be due to secondary role of this protein in physiological phenomenon like motility and capacitation and/or signaling pathway leading to oocyte activation. However, further research is needed to confirm these results.

In the next step, we assessed tr-KIT protein in sperm of infertile men with failed fertilization and globozoospermia. Relative expression of tr-KIT protein was significantly lower in infertile men with failed fertilization and globozoospermic individuals compared to fertile men. Therefore, low expression of tr-KIT could be along with many other proteins whose expression is reduced in infertile men with low or absence of fertilization. These data possibly could suggest that one reason of failed oocyte activation or failed fertilization in both infertile groups could be due to reduced expression of tr-KIT.

Considering that fertilization is a multifactorial process and many factors such as anomalies in the oocyte and/or sperm, chromatin damage, inability to activate the oocyte, failure in chromatin decondensation (6) and the technique used, could affect fertilization outcome, we assessed sperm DNA fragmentation in fertile individuals, and infertile men with either failed fertilization or globozoospermia. Sperm DNA damage that commonly due to oxidative stress has been associated with failure in fertilization, embryo quality as well as poor implantation, and pregnancy outcomes (40). As we expected, the percentage of fragmented DNA in the sperm of both infertile groups were higher compared to fertile men. In addition, we observed significant correlation between percentage of DNA fragmentation and fertilization rate. Therefore, individuals with high DNA fragmentation are likely to have low fertilization rates. Similar to Muciaccia et al. (26), we also observed a significant negative correlation between percentage of sperm DNA fragmentation and tr-KIT. Therefore, we suggest that sperm tr-KIT and DNA fragmentation could be considered as new markers for assessing human semen quality.

Conclusion

The result of this study clearly demonstrated that sperm tr-KIT has an important association with fertilization in humans, and its expression is decreased in individuals with low fertilization rate and globozoospermia. Taken together, further studies are requiring to reveal more light on involvement of tr-KIT in fertilization and to deepen our undemanding regarding the molecular mechanism of failed fertilization.

Acknowledgements

This study was financial supported by Royan institute and we would like to express our gratitude to staff of Isfahan Fertility and Infertility for their full support. We also thank Dr. R.A. Lockshin for critical review of the manuscript. None of the authors has any conflicts of interest to disclose.

Authors' Contributions

M.T.; Conception, design, collection and/or assembly of data, data analysis, interpretation, manuscript writing and final approval of manuscript. S.H.; Collection and preparation of samples and data, analysis of data. M.H.N.E.; Conception, design, data analysis, interpretation, manuscript writing and final approval of manuscript. Z.Z., A.S.; Interpretation, manuscript writing and final approval of manuscript. M.N.; Data analysis, read and approve of the final manuscript. All authors read and approved the final manuscript.

References

- 1. Henkel R. Sperm preparation: state-of-the-art—physiological aspects and application of advanced sperm preparation methods. Asian J Androl. 2012; 14(2): 260-269.
- Neri Q V, Lee B, Rosenwaks Z, Machaca K, Palermo GD. Understanding fertilization through intracytoplasmic sperm injection (ICSI). Cell Calcium. 2014; 55(1): 24-37.
- 3. Palermo G D, Neri QV, Takeuchi T, Rosenwaks Z. ICSI: where we have been and where we are going. Semin Reprod Med. 2009; 27 (2): 191-201.
- Sakkas D, Ramalingam M, Garrido N, Barratt CL. Sperm selection in natural conception: what can we learn from mother nature to improve assisted reproduction outcomes? Hum Reprod Update. 2015; 21(6): 711-726.
- Ebner T, Moser M, Sommergruber M, Jesacher K, Tews G. Complete oocyte activation failure after ICSI can be overcome by a modified injection technique. Hum Reprod. 2004; 19(8): 1837-1841.
- 6. Swain JE, Pool TB. ART failure: oocyte contributions to unsuccessful fertilization. Hum Reprod Update. 2008; 14(5): 431-446.
- Aarabi M, Balakier H, Bashar S, Moskovtsev SI, Sutovsky P, Librach C L, et al. Sperm-derived WW domain-binding protein, PAWP, elicits calcium oscillations and oocyte activation in humans and mice. FASEB J. 2014; 28(10): 4434-4440.
- Aarabi M, Balakier H, Bashar S, Moskovtsev SI, Sutovsky P, Librach CL, et al. Sperm content of postacrosomal WW binding protein is related to fertilization outcomes in patients undergoing assisted reproductive technology. Fertil Steril. 2014; 102(2): 440-447.
- 9. Aarabi M, Sutovsky P, Oko Ř. Re: Is PAWP the 'real'sperm factor? Asian J Androl. 2015; 17(3): 446-449.
- Kashir J, Nomikos M, Swann K, Lai FA. PLCζ or PAWP: revisiting the putative mammalian sperm factor that triggers egg activation and embryogenesis. Mol Hum Reprod. 2015; 21(5): 383-388.
- 11. Nomikos M, Swann K, Lai FA. Is PAWP the "real" sperm factor? Asian J Androl. 2015; 17(3): 444-446.
- Sette C, Bevilacqua A, Bianchini A, Mangia F, Geremia R, Rossi P. Parthenogenetic activation of mouse eggs by microinjection of a truncated c-kit tyrosine kinase present in spermatozoa. Development. 1997; 124(11): 2267-2274.
- Amdani SN, Yeste M, Jones C, Coward K. Sperm factors and oocyte activation: current controversies and considerations. Biol Reprod. 2015; 93(2): 50.
- Amdani SN, Yeste M, Jones C, Coward K. Phospholipase C zeta (PLCζ) and male infertility: clinical update and topical developments. Adv Biol Regul. 2016; 61: 58-67.
- 15. Sanders JR, Swann K. Molecular triggers of egg activation at fertilization in mammals. Reproduction. 2016; 152(2): R41-R50.
- Swann K, Lai FA. The sperm phospholipase C-ζ and Ca2+ signalling at fertilization in mammals. Biochem Soc Trans. 2016; 44(1): 267-272.
- 17. Swann K, Lai FA. Egg activation at fertilization by a soluble sperm protein. Physiol Rev. 2016; 96(1): 127-149.
- Anifandis G, Messini C I, Datopoulos K, Daponte A, Messinis IE. Sperm contributions to oocyte activation: more that meets the eye. J Assist Reprod Genet. 2016; 33(3): 313-316.
- Nomikos M, Kashir J, Swann K, Lai FA. Sperm PLCζ: from structure to Ca2+ oscillations, egg activation and therapeutic potential. FEBS Lett. 2013; 587(22): 3609-3616.
- Nomikos M. Novel signalling mechanism and clinical applications of sperm-specific PLCζ. Biochem Soc Trans. 2015; 43(3): 371-376.
- 21. Vadnais ML, Gerton GL. From PAWP to "Pop": opening up new pathways to fatherhood. Asian J Androl. 2015; 17(3): 443-444.
- Rossi P, Dolci S, Sette C, Geremia R. Molecular mechanisms utilized by alternative ckit gene products in the control of spermatogonial proliferation and spermmediated egg activation. Andrologia. 2003; 35(1): 71-78.
- Sette C, Bevilacqua A, Geremia R, Rossi P. Involvement of phospholipase Cγ1 in mouse egg activation induced by a truncated form of the c-kit tyrosine kinase present in spermatozoa. J Cell Biol. 1998; 142(4): 1063-1074.
- 24. Sette C, Paronetto MP, Barchi M, Bevilacqua A, Geremia R, Rossi

P. Trkitinduced resumption of the cell cycle in mouse eggs requires activation of a Srclike kinase. EMBO J. 2002; 21(20): 5386-5395.

- Rossi P, Sette C, Dolci S, Geremia R. Role of c-kit in mammalian spermatogenesis. J Endocrinol Invest. 2000; 23(9): 609-615.
- Muciaccia B, Sette C, Paronetto MP, Barchi M, Pensini S, D'agostino A, et al. Expression of a truncated form of KIT tyrosine kinase in human spermatozoa correlates with sperm DNA integrity. Hum Reprod. 2010; 25(9): 2188-2202.
- World Health Organization. WHO Laboratory Manual for the examination and processing of human semen. Geneva, Switzerland: WHO; 2010.
- Kheirollahi-Kouhestani M, Razavi S, Tavalaee M, Deemeh M R, Mardani M, Moshtaghian J, et al. Selection of sperm based on combined density gradient and Zeta method may improve ICSI outcome. Hum Reprod. 2009; 24(10): 2409-2416.
- Aghajanpour S, Ghaedi K, Salamian A, Deemeh M R, Tavalaee M, Moshtaghian J, et al. Quantitative expression of phospholipase C zeta, as an index to assess fertilization potential of a semen sample. Hum Reprod. 2011; 26(11): 2950-2956.
- Tavalaee M, NasrEsfahani MH. Expression profile of plcζ, pawp, and trkit in association with fertilization potential, embryo development, and pregnancy outcomes in globozoospermic candidates for intracytoplasmic sperm injection and artificial oocyte activation. Andrology. 2016; 4(5): 850-856.
- Chansel-Debordeaux L, Dandieu S, Bechoua S, Jimenez C. Reproductive outcome in globozoospermic men: update and prospects. Andrology. 2015; 3(6): 1022-1034.
- 32. Escoffier J, Yassine S, Lee HC, Martinez G, Delaroche J, Coutton C, et al. Subcellular localization of phospholipase C ζ in human sperm and its absence in DPY19L2-deficient sperm are consistent with its role in oocyte activation. Mol Hum Reprod. 2015; 21(2): 157-168.

- Taylor SL, Yoon SY, Morshedi MS, Lacey DR, Jellerette T, Fissore RA, et al. Complete globozoospermia associated with PLCζ deficiency treated with calcium ionophore and ICSI results in pregnancy. Reprod Biomed Online. 2010; 20(4): 559-564.
- Heytens E, Parrington J, Coward K, Young C, Lambrecht S, Yoon S Y, et al. Reduced amounts and abnormal forms of phospholipase C zeta (PLCζ) in spermatozoa from infertile men. Hum Reprod. 2009; 24(10): 2417-2428.
- Tavalaee M, Nomikos M, Lai FA, Nasr-Esfahani MH. Expression of sperm PLCζ and clinical outcomes of ICSI-AOA in men affected by globozoospermia due to DPY19L2 deletion. Reprod Biomed Online. 2018; 36(3): 348-355.
- Escoffier J, Lee HC, Yassine S, Zouari R, Martinez G, Karaouzène T, et al. Homozygous mutation of PLCZ1 leads to defective human oocyte activation and infertility that is not rescued by the WW-binding protein PAWP. Hum Mol Genet. 2016; 25(5): 878-891.
- Afiyani AA, Deemeh MR, Tavalaee M, Razi M, Bahadorani M, Shokrollahi B, et al. Evaluation of heat-shock protein A2 (HSPA2) in male rats before and after varicocele induction. Mol Reprod Dev. 2014; 81(8): 766-776.
- Liu Y, Jiang M, Li C, Yang P, Sun H, Tao D, et al. Human t-complex protein 11 (TCP11), a testis-specific gene product, is a potential determinant of the sperm morphology. Tohoku J Exp Med. 2011; 224(2): 111-117.
- Sette C, Dolci S, Geremia R, Rossi P. The role of stem cell factor and of alternative c-kit gene products in the establishment, maintenance and function of germ cells. Int J Dev Biol. 2000; 44(6): 599-608.
- Jin J, Pan C, Fei Q, Ni W, Yang X, Zhang L, et al. Effect of sperm DNA fragmentation on the clinical outcomes for in vitro fertilization and intracytoplasmic sperm injection in women with different ovarian reserves. Fertil Steril. 2015; 103(4): 910-916.

Relationship Study of The Verified Human Epidermal Growth Factor Receptor 2 Amplification with Other Tumor Markers and Clinicohistopathological Characteristics in Patients with Invasive Breast Cancer, Using Chromogenic In Situ Hybridization

Abdolazim Sarli, Ph.D.¹, Hossein Mozdarani, Ph.D.^{1*}, Nasser Rakhshani, M.D.², Sohail Mozdarani, M.Sc.³

Department of Medical Genetics, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
 Firoozgar Hospital, Gastrointestinal and Liver Diseases Research Center, Iran University of Medical Sciences, Tehran, Iran
 Cytogenome Medical Genetics Laboratory, Chamran Medical Building, Ale Ahmad Highway, Tehran, Iran

*Corresponding Address: P.O.Box: 14115-111, Department of Medical Genetics, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

Email: mozdarah@modares.ac.ir

Received: 17/July/2018, Accepted: 9/October/2018

Abstract

Objective: Human epidermal growth factor receptor 2 (*HER-2*), as a crucial factor involved in about 20% of breast cancer cases, is one of the most reliable tumor markers to determine prognosis and therapeutic trend of this disease. This marker is generally assessed by immunohistochemistry (IHC) technique. In the cases that result of IHC test cast doubt (+2), the test should be repeated or validated by applying in situ hybridization techniques, like chromogenic in situ hybridization (CISH). In this regard, the goal of current study was to figure out the link between different clinicopathological characteristics of patients suffering from invasive breast cancer, using tumor markers, hormone receptor (HR) and HER-2. Comparing IHC and CISH techniques for evaluating diagnostic value and usefulness of HER-2 were also the other objective of this study.

Materials and Methods: Based on this retrospective study, histological markers of 113 individuals suffering from invasive breast cancer -such as estrogen receptor (ER), progesterone receptor, *HER-2* receptor, E-cadherin, CK5/6, vimentin and Ki67 were examined by IHC technique. HER-2 amplification of all patients was also evaluated by CISH. Clinicopathological information of the patients was also extracted from medical documents and their associations with tumor markers were statistically evaluated.

Results: There is a significant relationship between tumor size, CK5/6 and tumor grade with HR status. Similar relationship was observed between *HER-2* status and HR status, as well as vascular invasion (P<0.05). The comparison of *HER-2* amplification showed no complete concordance of the result obtained from these two techniques, with score +3.

Conclusion: Since the status of *HER-2* is very important in decision making of the treatment process, CISH technique is recommended in the malignant conditions as the primary test, instead of IHC. In this study, we also determined that *HER-2* expression is greatly correlated with ER- and PR- status. This might propose a better prognosis for *HER-2*+ patients.

Keywords: Breast Cancer, Chromogenic In Situ Hybridization, HER-2, Tumor Markers

Cell Journal(yakhteh), Vol 21, No 3, October-December (Autumn) 2019, Pages: 322-330 _

Citation: Sarli A, Mozdarani H, Rakhshani N, Mozdarani S. Relationship study of the verified human epidermal growth factor receptor 2 amplification with other tumor markers and clinicohistopathological characteristics in patients with invasive breast cancer, using chromogenic in situ hybridization. Cell J. 2019; 21(3): 322-330. doi: 10.22074/cellj.2019.6219.

Introduction

Breast carcinoma is a multifactorial ailment comprised of noticeable biological subtypes with vast variation in clinical, pathological and molecular features having various prognostic and therapeutic implications. The nature of this malignancy is interconnected with its clinical outcomes (1). It is important to note that up to 21 distinct histological subtypes and at least four various molecular subtypes of breast cancer, correlating with distinct risk factors, have thus far been diagnosed which are biologically different in presentations and results (2, 3).

Evaluating different biological markers -including presence or absence of hormone (i.e. estrogen or progesterone) receptors (named respectively HR⁺ or HR⁻) and excessive level of human epidermal growth factor receptor 2 (*HER*-2)- is the most applicable method for identifying the subtype of the cancer (4), leading to classification of some distinct subtypes of breast cancer: luminal A (HR⁺/*HER*-2⁻), triple negative (HR⁻/*HER*-2⁻), luminal B (HR⁺/*HER*-2⁺) and *HER*-2⁻enriched (HR⁻/*HER*-2⁺) tumors (5). *HER-2* gene product is a 185-kDa trans-membrane growth factor receptor with tyrosine kinase activity involved in cellular signaling. It is responsible for regulating cell growth and development (6). Clinical studies show that *HER-2* gene is amplified in 20-30% of all breast cancers (7), out of which overexpression is the direct result of this gene amplification in ~90-95% of cases (6). This phenomenon is a remarkable prognosis factor associated with lymph node metastasis, HR⁻ tumors, high-grade tumor, great recurrence risk after operation, weak response to common chemotherapy and no chance of long-term survival (8).

HER-2 expression is an important factor in therapeutic decision-making of breast cancer, since HER-2 protein (*HER-2* gene product) is targeted for specific treatment by humanized recombinant monoclonal antibody Trastuzumab. So that, this drug could only be applied for treatment of patients with *HER-2*⁺ malignancy (9).

These days, expression of estrogen receptor (ER),

progesterone receptor (PR) and HER-2 are measured by immunohistochemistry (IHC) technique, as a prognostic factor applied in the routine protocol of breast cancer treatment. In this technique, amplification of HER-2 is reported in three scores: i. No amplification of the targeted gene which is considered as +1, ii. An interface that does not indicate whether there is any increase in the HER-2 protein level and it is shown as +2, in addition to iii. The definite amplification of HER-2 which is considered as +3. The patient's IHC scored +2 should be rechecked by IHC or evaluated by some in situ hybridization techniques, like fluorescent in situ hybridization (FISH) or chromogenic in situ hybridization (CISH). Some studies implicate that CISH is more sensitive than IHC (10).

In this study, we examined sensitivity of the results obtained from IHC and CISH tests. For this purpose, HR (ER and PR) and HER-2 proteins of breast cancer patients were evaluated by these two techniques. In addition, all demographic and histopathological characteristics of the patients were recorded. The results of IHC test were scored as +1, +2 and +3, and compared to CISH test representing status of HER-2 expression (HER-2⁺ and HER-2⁻ groups). Finally, histopathological characteristics and tumor subtypes obtaining from these two techniques were analyzed to detect meaningful correlations.

Materials and Methods

This retrospective study was conducted over a period of four years at Mehr Hospital Pathology Department (Tehran, Iran). Over this time, 113 mastectomy specimens were obtained. In all cases, clinical features and tumor studies, including ER, PR, E-cadherin, CK5/6, vimentin and Ki67, as well as HER-2, were performed on formalin-fixed paraffinembedded (*FFPE*) tissue samples. Disease of specimens was completely gross based on a standard protocol. In addition, other data including tumor size, side of the breast, invasive ductal or lobular carcinoma, in situ component, grade and tumor vascular invasion were recorded.

Tissue was subjected to routine processing and sections were stained with hematoxylin and eosin stain (11). The histopathological criteria were diagnosed based on WHO classification and the samples were graded, applying Modified Blooms Richardson Grading System. In addition, antibodies were applied to ER, PR, *HER-2* receptor, E-cadherin, CK5/6, vimentin and Ki67.

Evaluation of progesterone receptor, estrogen receptor and HER-2 using IHC

Slices were made in thicknesses of 3-4 micrometers and placed on polyethylene lysine-coated slides. They were next deparaffined in xylene followed by distilling off with ethanol. Paraffin and healing slices were next set in 3% hydrogen peroxide solution (Sigma-Aldrich, USA). Antigenic reagents were performed by a 0.01 M citrate buffer solution with pH=6 for 20 minutes in microwave. In the next step, the sections were separately incubated with 7 antibodies (all from AbCam, UK) for 60 minutes at 37°C: Monoclonal Mouse Anti-ErbB2 Affibody® Molecule, Monoclonal Mouse Antihuman Estrogen, Monoclonal Mouse Antihuman Progesterone, Monoclonal Mouse Anti-E Cadherin antibody,

Monoclonal Mouse Anti-Cytokeratin 5+6 antibody (D5/16 B4), Monoclonal Mouse Anti-vimentin antibody and Monoclonal Mouse Anti-Ki67 antibody. Normal tissue surrounding the tumor was used as the control of HER-2, ER and PR. We could also quantify ER, PR staining by utilizing Allred score. All the slides were quantified by giving proportional scores regarding the percentage of cells, nuclear stain presence and intensity score considering the intensity of staining. The proportional score (PS) is as follows: 1% of cells representing nuclear stain, 10% of cells demonstrating nuclear stain, 33% of cells showing nuclear stain, 66% of cells expressing nuclear stain, 100% of cells showing nuclear stain. Intensity score (IS) is as follows: 0-negative weak staining, 1- intermediate staining and 2- strong staining. Total score (TS) is considered as follow: sum of PS+intensity. TS greater than 2 is regarded positive for significant expression of ER and PR. Immunohistochemical assessment of HER-2 overexpression was considered positive, considering more than 10% of cells is severely stained (+3 score). In ambiguous cases (+2 score), they had to be confirmed by CISH.

Chromogenic in situ hybridization

In this experiment, paraffin blocks were divided into 5-6 micron sections (at least 2 sections) to evaluate expression of *HER-2* marker. We also categorized all original breast tumor tissues with either modified radical mastectomy or breast-conserving surgery to confirm diagnosis of the invasive carcinomas.

The test has been conducted by applying CISH, based on Zyto Dot: 2C SPEC HER-2/CEN-17 dual Probes Kit protocol (Zytovision, Germany). The PD-12 probe contains digoxigenin-labeled polynucleotides targeting sequences of the *HER-2* gene and DNA-labeled polynucleotides targeting alpha-satellites of the centromere of chromosome 17 causing formation of green and red signals, illustrated by light microscopy (×40 objective lens). All of these reactions were performed in two days, following four steps, in line with the kit protocol (www.zytovision.com).

CISH hybridization signal of one single copy of HER-2 gene, appears like a distinct dark green dot-shaped signal, while the signal of one single copy of chromosome 17 centromeric region appears as a distinct bright red dotshaped signal which can clearly be distinguished from the background counterstained with hematoxylin (Fig.1). All slides were analyzed and the results were recorded and scored in accordance to the American Society of Clinical Oncology/ College of American Pathologists (ASCO/CAP) guidelines. Briefly, the numbers of CEN-17 and HER-2 signals were counted in 100 non-overlapping invasive cancer cell nuclei, applying at least three distinct tumor fields (when possible). *HER-2* signal heterogeneity was not regarded in this study. Where the mean *HER-2*/CEN-17 ratio in any field is 2 or greater, the tumor is, therefore, amplified. Where the ratio is less than 2 whereas average of *HER-2* signal number per cell is equal to or less than 2, it is not amplified. Cases with a ratio of less than 2 and HER-2 signal number per cell between 4 and 6 were considered as equivocal borderline results and after counting an additional 20 nuclei according to new ASCO/CAP guideline 2018 version (12, 13), final decision on the degree of amplification was made.



Fig.1: Illustration of a CISH result performed on the patient tumor sample. **A.** HER-2⁺ sample, whereby green-to-red ratio is more than 2 and **B.** HER-2⁻ sample, whereby green-to-red ratio is less than 2 (red color is the indicator of chromosome 17 centromeric probes, and green color is the indicator of specified probes of *HER*-2 gene) (scale bar: 100 μ m).

Ethical considerations

All experiments were performed in accordance with relevant guidelines and regulations. All FFPE samples were obtained from the Mehr Hospital. This study was approved by the Ethics Committee of Tarbiat Modares University (registered number: 52D/4922), Tehran, Iran. Written informed consent was obtained from each participant before FFPE sample collection.

Statistical analysis

In this study, chi-square data analysis and Fisher's exact test were applied. All statistical analyses were conducted using Statistics Package for Social Sciences (SPSS) version 18 at the significant level of P<0.05. Quantitative variables were reported as mean \pm SD and qualitative variables were also reported as frequency (%).

Results

In the present study, various clinicopathological parameters in 113 cases of infiltrating ductal (102 cases) and lobular (11 cases) carcinoma were analyzed and summarized in Table 1. The range of breast cancer patient age onset was between 27 and 95 years old. Demographic data is given in Table 1.

The ER and PR relationship with pathological and demographic features, as well as clinical characteristics of patients are presented in Table 2.

Table 1: Demographic characteristics of ductal carcinoma breast cancer	•
patients	

Characteristics	Number of subjects (%)
Age (Y)	
Mean	54.05 ± 12.729
Range	27-95
Stage at diagnosis	
Stage I	37 (32.7)
Stage II	28 (24.7)
Stage III	27 (23.8)
Not determined	21 (18.5)
Breast involvement	
Right breast	67 (59.3)
Left breast	42 (37.5)
Bilateral involvement	4 (3.2)
Size of tumor	
More than 2 cm	81 (71)
Less than 2 cm	32 (29)
Grade of tumor	
Grade 1	25 (22.1)
Grade 2	57 (50.4)
Grade 3	22 (19.4)
Not determined	9 (7.9)
Vascular invasion	57 (51.8)
Type of breast cancer	
Ductal carcinoma	101 (90.2)
Lobular carcinoma	9 (8.0)
In situ component of tumor	57 (50.9)
Hormone receptor status (IHC)	
\mathbf{ER}^+	85 (75.9)
ER	27 (24.1)
PR ⁺	69 (61.6)
PR-	43 (38.4)
ER ⁻ and PR ⁻	30 (27)
HER-2	
+1 (negative)	27 (24.1)
+2 (equivocal)	65 (58.0)
+3 (positive)	21 (17.9)
Biomarkers	
E-cadherin positive	33 (68.8)
CK5/6 positive	9 (14.1)
Vimentin positive	4 (7.3)
Ki67	92 (95.8)
HER-2 (CISH)	
Amplified	35 (31.3)
Not amplified	77 (68.8)
Triple negative	18 (16.1)

PR; Progesterone receptor and ER; Estrogen receptor.

Table 2: Comparison of biomarker, demographic and clinical variables in terms of different combinations of ER and PR

Variable	ER ⁻ /PR ⁺ or ER ⁺ /PR ⁻	ER ⁺ /PR ⁺	ER ⁻ /PR ⁻	P value (Chi-square test)
Age				
≤45	7 (41.2)	17 (26.2)	5 (16.7)	0.18
>45	10 (58.8)	48 (73.8)	25 (83.3)	
Tumor size				
≤2	13 (76.5)	26 (40.0)	7 (23.3)	0.002
>2	4 (23.5)	39 (60.0)	23 (76.7)	
Breast				
Right	12 (70.6)	36 (55.4)	19 (63.3)	0.52
Left	4 (23.5)	28 (43.1)	10 (33.3)	
Bilateral	1 (5.9)	1 (1.5)	1 (3.3)	
Invasive ductal carcinoma				
No	1 (5.9)	7 (10.8)	3 (10.0)	0.91
Yes	16 (94.1)	58 (89.2)	27 (90)	
Invasive lobular carcinoma				
No	17 (100.0)	58 (89.2)	28 (100.0)	0.38
Yes	0 (0.0)	7 (10.8)	0 (0.0)	
In situ component				
No	7 (41.2)	30 (46.2)	18 (60.0)	0.35
Yes	10 (58.8)	35 (53.8)	12 (40.0)	
Grade				
1	5 (31.3)	18 (28.6)	2 (8.0)	0.01
2	10 (62.5)	35 (55.6)	12 (48.0)	
2	1 (6.3)	10 (15.9)	11 (44.0)	
Vascular invasion				
Negative	7 (41.2)	34 (52.3)	12 (42.9)	0.57
Positive	10 (58.8)	31 (47.7)	16 (57.1)	
Stage				
Ι	2 (66.7)	11 (55.0)	4 (44.4)	0.73
II	0 (0.0)	6 (30.0)	2 (22.2)	
III	1 (33.3)	3 (15.0)	3 (33.3)	
E-cadherin				
Negative	3 (42.9)	10 (35.7)	2 (15.4)	0.38
Positive	4 (57.1)	18 (64.3)	11 (84.6)	
CK5/6				
Negative	1 (10.0)	2 (5.9)	14 (70.0)	0.04
Positive	9 (90.0)	32 (94.1)	6 (30.0)	
Vimentin				
Negative	6 (100.0)	33 (97.1)	12 (80)	0.11
Positive	0 (0.0)	1 (2.9)	3 (20.0)	
Ki67				
Negative	0 (0.0)	3 (5.3)	1 (4.0)	0.83
Positive	13 (100)	54 (94.7)	24 (96.0)	

PR; Progesterone receptor and ER; Estrogen receptor. Data are presented as n (%).

According to Table 2, only association of CK5/6 with different combinations of ER and PR results is statistically noticeable (P<0.05). There is no significant association of E-cadherin, vimentin and Ki67 clinical variables with different combinations of ER and PR results (P>0.05). Chi-square analyses also indicate no significant association of tumor size and grade variables with different combinations of ER and PR (P<0.05). On the other hand, one of the goals of this study was to investigate potential association of *HER-2* status (positive or negative result) using CISH technique with pathological and clinical variables of the patients. Results of this objective are reported in Tables 3 and 4.

Table 3: Comparison of histological variables in patients	with positive and
negative CISH HER-2 result	

Variable	CISH	CISH <i>HER-2</i> P value (Chi-squar			
	Positive	Negative			
ER					
Positive	20 (57.1)	59 (76.6)	0.03		
Negative	15 (42.9)	18 (23.4)			
PR					
Positive	16 (45.7)	52 (67.5)	0.02		
Negative	16 (54.3)	25 (32.5)			
E-cadherin					
Positive	11 (78.6)	22 (64.7)	0.34		
Negative	3 (21.4)	12 (35.3)			
CK5/6					
Positive	2 (10.0)	7 (15.9)	0.70		
Negative	18 (90.0)	37 (84.1)			
Vimentin					
Positive	0 (0.0)	4 (10.8)	0.14		
Negative	18 (100.0)	33 (89.2)			
Ki67					
Positive	29 (96.7)	62 (95.4)	0.14		
Negative	1 (3.3)	3 (4.6)			

Data are presented as n (%).

Table 4: Comparison of demographic and clinical variables in	patients
with positive and negative CISH HER-2 result	

Variable	CISH	HER-2	P value
			(Chi-square test)
	Positive	Negative	
Age			
≤45	8 (20.0)	22 (28.6)	0.33
>45	28 (80.0)	55 (71.4)	
Tumor size			
≤2	13 (37.1)	33 (42.9)	0.56
>2	22 (62.9)	44 (57.1)	
Breast			
Right	25 (71.4)	42 (54.5)	0.21
Left	9 (25.7)	33 (42.9)	
Bilateral	1 (2.9)	2 (2.6)	
Invasive ductal carcinoma			
Yes	34 (97.1)	67 (87.0)	0.09
Invasive lobular carcinoma			
Yes	1 (2.9)	8 (13.0)	0.17
In situ component			
Positive	18 (51.4)	39 (50.6)	0.93
Negative	17 (48.6)	38 (49.4)	
Grade			
1	5 (14.7)	20 (28.6)	0.30
2	21 (61.8)	36 (51.4)	
3	8 (23.5)	14 (20.0)	
Vascular invasion			
Positive	25 (71.4)	32 (42.7)	0.005
Negative	10 (28.6)	43 (57.3)	
Stage			
I	6 (40 0)	11 (64 7)	0.08
П	3(20.0)	5 (20 4)	0.00
ш	5(20.0) 6(40.0)	1(5.9)	
III	6 (40.0)	1 (5.9)	

Data are presented as n (%).

According to Table 4, results obtained from chi-square analysis revealed that only association of vascular invasion with CISH *HER-2* status is statistically significant (P<0.05). Finally, in order to detect *HER-2* amplification, sensitivity and specificity of CISH were compared to IHC technique. The results are illustrated in Figure 2.

As shown in Figure 2, there are differences in *HER*-2 amplification frequency of +2 and +3 scores between CISH and IHC methods. In the cases of +1 score (i.e. *HER*-2 negative) using IHC, the results were confirmed by CISH technique. But, in the cases of +2 and +3 scores using IHC (i.e. *HER*-2 positive), CISH technique reveals contradictory cases.



Fig.2: Comparison of two techniques, CISH and IHC, in HER-2 amplification detection. HER2 status is defined according to IHC in 3 states: +1 (HER2 negative) where the cell membranes are not stained or less than 10% of the cells are stained. +2 (Equivocal), in which the membrane contains more than 10% of the cells weakly or moderately stained and +3 (HER2 positive) in which the membrane of more than 10% of the cells is completely and severely stained.

Discussion

Breast cancer is the main cause of around 9-34% of all patient malignancies in women, and about 1 million new cases are recognized annually around the world (14). In addition, breast cancer is a widespread type of malignancy occurring in women living in developed countries and it is the fifth cause of death among all cancers (14, 15). One of the important issues in diagnosis and treatment of breast cancer is impossibility of early detection (16). Therefore, improving diagnostic process will have a remarkable output in the consequences of breast cancer.

Prognostic and diagnostic factors play important role in several aspects, including perception of the disease process in patients, predicting disease outcome, choosing the right treatment and planning for implementation of the extra treatment process. ER and PR status determination is very important in choosing the right treatment of breast cancer (17). These receptors are also considered as prognostic factors during hormone therapy (18).

In our study, the onset age mean of patients is 54 which, to some extent, is higher than the patients in other studies. In a study performed by Erbil et al. (19) age mean of the total number of 231 patients was 45 years and in the other study carried out by Mohaghegh et al. (20), the reported average of age was 48.3 years. In a study conducted by Payandeh et al. (21), the age mean of patients was 46.39 years.

In our study, 85 (75.9%) of the total cases were ER⁺, while 69 (61.6%) of them were PR⁺. Therefore, in our population study, ER⁺ patients have considerable prevalence and in comparison with other studies, the number of ER⁺ individuals is greater than that of PR⁺ (22, 23).

There are controversial reports on association of HR with clinicopathological features of the patients. In our study, no significant association between ER and PR with pathological features of E-cadherin, vimentin and Ki67 was observed. Furthermore, although there was a correlation between tumor size and grade of the disease, in addition to CK5/6, we did not notice a remarkable link between HR status and clinical features as well as demographic information including age, stage of disease, invasive lobular and in situ component. Thike et al. (24) showed that there is no association between age and HR status. However, in another study performed by Jalava et al. (23) an association between age and ER status was determined. Jalava et al. (23) and Aaltomaa et al. (25) showed no specific relationship between tumor size and HR status. However, in this study, we determined that size of the tumor in HR⁺ patients was more than 2 cm. Moreover, Moreover, in a study a lack of correlation has been reported between HR and histological analysis of carcinoma cells, while in several studies a correlation between HR⁺ and invasive lobular cancer was reported. HR⁺ status is generally common in patients with low tumor stage according to the result obtained from our study. However, due to the lack of samples with diagnosed stage of disease, it was not statistically significant. Our results also indicate that HR⁺ tumors have more +2 score than HR⁻ tumors. This indication is in line with several, but not all, studies (23). The basal type cytokeratin CK4/5 expression correlates with poor prognostic features, such as early recurrence, axillary lymph node positivity, high tumor grade, Ki-67 positivity and ER negativity (25). Our results showed that CK4/5 is often seen in HR⁻ samples, in accordance with those of Choccalingam et al. (26) reports who also demonstrated that basal-like breast cancer expression, defined by basal cytokeratin expression, correlates with negative hormonal status and shorter disease-free intervals. Trastuzumab drug is used to treat patients suffering from HER-2+ invasive breast cancer tumors. In HER-2⁻ cases, however, administration of this drug not only fails to have any benefit for the patients, but also it results in cardiotoxicity and additional costs for patients (27).

In the present study, 35 (31%) patients showed overexpression of *HER*-2. The worldwide prevalence of women with *HER*-2⁺ breast cancer is 15-20% of the total affected cases which is also related to invasive forms of the disease (12). *HER*-2⁺ cancer cells can produce two millions copy of the relevant protein on their surfaces which is almost 100 fold more than normal cells. This

promotes the cancer cells to grow and reproduce faster. An essential step in the signaling pathway leading to cancer cell growth is the dimerization of the *HER-2* receptor protein (28). Several studies have reported the relationship between *HER-2* and prognostic factors (29). In a research study, Konecny et al. (30) showed a reverse relationship of *HER-2* with ER and PR status. Additionally, in a cohort study, a reverse correlation of *HER-2* with HR status as well as a positive correlation between tumor grade and overexpression of *HER-2* was reported (31).

In our study, most of the HER-2⁺ patients were aged more than 45 years old. According to our results, HER-2 showed a significant relationship with tumor vascular invasion; in most of the HER-2⁺ patients, tumor also had vascular invasion, while in the case of HER-2⁻ patients, vascular invasion showed no statistical difference. Other prognostic factors related to breast cancer showed no statistical relationship with HER-2 status. In this study, HER-2 gene expression significantly associated with ER⁻ and PR⁻ status. This is similar to the study of Ariga et al. (32). It has been recommended that this association could reflect a better prognosis. However, the other studies revealed that ER⁺/HER-2⁺ status accompanied with a poorer survival rate than $ER^+/HER-2^-$ status. Therefore, it sounds that *HER-2* expression is a better predicator of response to hormonal therapy than ER status itself.

Whereas these results are in accordance with previous studies, more sample size and clinicopathological information is needed to reach more precise and comprehensive results. In this way, individuals who are candidates for *HER-2* examination, in the process of treatment could be diagnosed at the early stage of disease using CISH technique, with no need of IHC technique application.

In this study, we analyzed the frequency of patient sample features by IHC and CISH methods. As mentioned previously, the results of 18 (16.1%) patients, analyzed by IHC and CISH techniques, were triple negative and 30 (27%) patients were ER⁻ and PR⁻ synchronously. However, this finding contradict with the previously reported frequency of triple negative breast cancer patients 54.83% among infiltrating ductal carcinoma. In addition, Sandhu et al. (33) in another study reported 31% prevalence of triple negative breast cancer in 7223 of Indian patients.

As mentioned before, we used CISH technique in our study. In *HER-2* examination, one of the remarkable privileges of CISH over IHC is the increase of specificity and sensitivity. The other advantage of in situ hybridization method for *HER-2* is that this examination be done through a comparative way with a reference sequence in one reaction on a slide which results in reduction of errors and increase of accuracy. Relatively qualitative method is another limitation of IHC technique, leading to inaccuracy of +1, +2 and +3 scores distinction related to HER-2. Moreover, this method is affected by technical errors, especially experience of operator (34).

In the case of solid tumors, CISH is better and the relative slides could be conserved longer, compared to FISH method. Additionally, detection of gene amplification is more beneficial using CISH in contrast to FISH, regarding that: i. In permanent staining, samples can be archived, ii. Bright field microscopy application would be feasible, iii. Identification of the target cells is easy, and iv. Tumor heterogeneity is easily assessed (35). In this study, we compared the results of CISH with IHC tests by examining a number of breast cancers.

Herceptin is an antibody-based drug utilized to treat breast cancer, by targeting overexpression of HER-2 protein, as it is observed in about one-third of breast cancer patients. Therefore, Herceptin is prescribed for HER-2⁺ patients. On the other hand, prescribing this medication for patients who are not diagnosed with conclusive *HER*-2 gene expression may lead to adverse side-effects and even faster disease progression as well as economically imposing high costs to the patients' family and public health system. Usually, +1 score is considered as nonamplification of HER-2 in IHC tests.

Currently, IHC tests are performed on most patients with breast cancer referring to laboratories in order to test for ER, PR, E-cadherin, CK5/6, vimentin and Ki67, among which HER-2 gene amplification is examined to prescribe and use Herceptin. In IHC technique for *HER-2* is classified to +1, +2, and +3 scores. While the +1 score is considered as HER-2 non-amplified class, the +3 score is considered as definitely amplified HER-2. The +2 score is considered as equivocal, meaning that there is uncertainties in the *HER-2* expression of patients. Therefore, either IHC tests should be repeated or the sample evaluation should be validated by FISH or CISH test (10), imposing more costs and time consequently. As previously mentioned, definitive answer to the HER-2 status is crucial for making decision to prescribe Herceptin.

In this study, we also compared the results of HER-2 amplifications by IHC and CISH techniques. According to results, CISH technique is considered more reliable than IHC. This comparisons show that only the cases with +1 score is considered non-amplified in IHC, fully validated by CISH method. Interestingly, the +2 score, which are considered equivocal results, account for 58% (65 patients) of all cases. In other words, only less than half of the patients receive the ultimate result using this test and their results must be verified by repeating experiment or utilizing other techniques such as CISH or FISH. Therefore, despite cheaper cost of IHC technique, it seems that would be a more rational to perform CISH test in patients from the beginning. It is worthy to note that $HER-2^+$ patients with +2 score results (74% of the cases) were verified through CISH method.

The results obtained from CISH test showed 2 patients, out of 20 *HER*- 2^+ cases with +3 IHC score, were actually *HER*- 2^- . False positivity of these 2 patients, as a significant IHC problem to test HER-2 protein overexpression, might lead to wrong process of their disease treatment. A minority of cases of breast cancer scoring *HER-2* (+3) by IHC using Herceptin test may not be associated with findings obtained from CISH, which confirms that the CISH technique has a higher accuracy and sensitivity (36).

In this project, we also calculated the rate of similarity between IHC and CISH results from two aspects: i. Proportion of the negative (+1) or positive (+3) cases obtained from IHC, to CISH and ii. Proportion of the cases identified as +2 IHC, to the CISH HER- 2^+ . Considering all these results, the rate of similarity between IHC and CISH in the cases of +1 and +3 scores was around 95.8% (45/47) and the concordance between +2 score and positive cases of CISH were around 26.2% (17/65). This result may be due to polysemy of chromosome 17 in breast tumors which may lead +2 IHC score of the cases to show false positive (37, 38). Totally, the overall concordance of these two techniques for detecting HER-2⁺ tumors is about 61%, while in the other studies, this concordance was varied from 52 to 82% (39). In other studies, the relationship between results of FISH/CISH techniques and IHC techniques has been reported. For instance, in a study performed by Bahreini et al. (40), it was demonstrated that 36% of +2 IHC score cases, identified by FISH technique, were positive and 64% were negative.

Conclusion

Since the results of HER-2 status is important for making decision of the treatment process, CISH technique is recommended to test *HER-2* expression in the malignant and invasive conditions rather than IHC. Additionally, in the presented study, *HER-2* expression was significantly linked to ER⁻ and PR⁻ status that may reflect a better prognosis.

Acknowledgments

This work was financially supported by Research Department of the Faculty of Medical Sciences, Tarbiat Modares University. There is no conflict of interest in this study.

Authors' Contributions

A.S., H.M., N.R.; Participated in the study design, data collection and evaluation, drafting manuscript and statistical analysis. S.M., A.S.; Set up IHC and CISH Techniques. A.S., H.M.; Contributed in the data interpretation and conclusion. All authors performed editing and approved the final version of this manuscript for submission. They also participated in the finalization of the manuscript and approved the final draft.

References

1. Ayadi L, Khabir A, Amouri H, Karray S, Dammak A, Guermazi M, et al. Correlation of HER-2 over-expression with clinico-patholog-

ical parameters in Tunisian breast carcinoma. World J Surg Oncol. 2008; 6: 112.

- Dieci MV, Orvieto E, Dominici M, Conte P, Guarneri V. Rare breast cancer subtypes: histological, molecular, and clinical peculiarities. Oncologist. 2014; 19(8): 805-813.
- Tamimi RM, Colditz GA, Hazra A, Baer HJ, Hankinson SE, Rosner B, et al. Traditional breast cancer risk factors in relation to molecular subtypes of breast cancer. Breast Cancer Res Treat. 2012; 131(1): 159-167.
- Kohler BA, Sherman RL, Howlader N, Jemal A, Ryerson AB, Henry KA, et al. Annual report to the nation on the status of cancer, 1975-2011, featuring incidence of breast cancer subtypes by race/ethnicity, poverty, and state. J Natl Cancer Inst. 2015; 107(6): djv048.
- Nosrati H, Mozdarani H, Hadad P, Omranipour R, Mozdarani S, Bakhshandeh M, et al. Inherent radiation sensitivity of lymphocytes of triple negative (TN) and luminal a: a comparison between patients with breast cancer and normal individuals as assayed by the micronucleus test. Arch Breast Cancer. 2017; 4(1): 10-15.
- Pauletti G, Godolphin W, Press MF, Slamon DJ. Detection and quantitation of HER-2/neu gene amplification in human breast cancer archival material using fluorescence in situ hybridization. Oncogene. 1996; 13(1): 63-72.
- Powell WC, Hicks DG, Prescott N, Tarr SM, Laniauskas S, Williams T, et al. A new rabbit monoclonal antibody (4B5) for the immunohistochemical (IHC) determination of the HER2 status in breast cancer: comparison with CB11, fluorescence in situ hybridization (FISH), and interlaboratory reproducibility. Appl Immunohistochem Mol Morphol. 2007; 15(1): 94-102.
- Dendukuri N, Khetani K, McIsaac M, Brophy J. Testing for HER2positive breast cancer: a systematic review and cost-effectiveness analysis. CMAJ. 2007; 176(10): 1429-1434.
- Pegram MD, Lipton A, Hayes DF, Weber BL, Baselga JM, Tripathy D, et al. Phase II study of receptor-enhanced chemosensitivity using recombinant humanized anti-p185HER2/neu monoclonal antibody plus cisplatin in patients with HER2/neu-overexpressing metastatic breast cancer refractory to chemotherapy treatment. J Clin Oncol. 1998; 16(8): 2659-2671.
- Tanner M, Gancberg D, Di Leo A, Larsimont D, Rouas G, Piccart MJ, et al. Chromogenic in situ hybridization: a practical alternative for fluorescence in situ hybridization to detect HER-2/neu oncogene amplification in archival breast cancer samples. Am J Pathol. 2000; 157(5): 1467-1472.
- Fischer AH, Jacobson KA, Rose J, Zeller R. Hematoxylin and eosin staining of tissue and cell sections. CSH Protoc. 2008; 2008: pdb. prot4986.
- Wolff AC, Hammond ME, Hicks DG, Dowsett M, McShane LM, Allison KH, et al. Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. J Clin Oncol. 2013; 31(31): 3997-4013.
- Wolff AC, Hammond MEH, Allison KH, Harvey BE, McShane LM, Dowsett M. HER2 testing in breast cancer: American society of clinical oncology/college of American pathologists clinical practice guideline focused update summary. J Oncol Pract. 2018; 14(7): 437-441.
- Curado MP. Breast cancer in the world: incidence and mortality. Salud Publica Mex. 2011; 53(5): 372-384.
- Mozdarani H. Breast cancer and paradigm of genomic instability. Arch Breast Cancer. 2016; 3(4): 102-105.
- Subramanian S, Keating NL. Delays in breast cancer diagnosis after a state policy limiting Medicaid enrollment. Cancer. 2017; 123(17): 3219-3221.
- Salimi M, Mozdarani H, Majidzadeh K. Expression pattern of ATM and cyclin D1 in ductal carcinoma, normal adjacent and normal breast tissues of Iranian breast cancer patients. Med Oncol. 2012; 29(3): 1502-1509.
- Paik S, Tang G, Shak S, Kim C, Baker J, Kim W, et al. Gene expression and benefit of chemotherapy in women with node-negative, estrogen receptor-positive breast cancer. J Clin Oncol. 2006; 24(23): 3726-3734.
- Erbil N, Dundar N, Inan C, Bolukbas N. Breast cancer risk assessment using the Gail model: a Turkish study. 2015. Asian Pac J Cancer Prev. 2015; 16(1): 303-306.
- Mohaghegh P, Yavari P, Akbari ME, Abadi A, Ahmadi F. The correlation between the family levels of socioeconomic status and stage at diagnosis of breast cancer. Iran J Cancer Prev. 2014; 7(4): 232-238.
- 21. Payandeh M, Sadeghi M, Sadeghi E, Janbakhsh A. Is there any concordance between of IHC with FISH in HER2-positive breast

cancer patients? Int J Hematol Oncol Stem Cell Res. 2017; 11(1): 43-48.

- Rajan G, Culas TB, Jayalakshmy PS. Estrogen and progesterone receptor status in breast cancer : a cross-sectional study of 450 women in Kerala , South India. World J Surg Oncol. 2014; 12: 120.
- Jalava P, Kuopio T, Huovinen R, Laine J, Collan Y. Immunohistochemical staining of estrogen and progesterone receptors: aspects for evaluating positivity and defining the cutpoints. Anticancer Res. 2005; 25(3c): 2535-2542.
- Thike AA, Chng MJ, Fook-Chong S, Tan PH. Immunohistochemical expression of hormone receptors in invasive breast carcinoma: correlation of results of H-score with pathological parameters. Pathology. 2001; 33(1): 21-25.
- Aaltomaa S, Lipponen P, Eskelinen M, Kosma VM, Marin S, Alhava E, et al. Hormone receptors as prognostic factors in female breast cancer. Ann Med. 1991; 23(6): 643-648.
- Choccalingam C, Rao L, Rao S. Clinico-pathological characteristics of triple negative and non triple negative high grade breast carcinomas with and without basal marker (CK5/6 and EGFR) expression at a rural tertiary hospital in India. Breast Cancer (Auckl). 2012; 6: 21-29.
- Ji Y, Sheng L, Du X, Qiu G, Chen B, Wang X. Clinicopathological variables predicting HER-2 gene status in immunohistochemistryequivocal (2+) invasive breast cancer. J Thorac Dis. 2014; 6(7): 896-904.
- Lewis Phillips GD, Li G, Dugger DL, Crocker LM, Parsons KL, Mai E, et al. Targeting HER2-positive breast cancer with trastuzumab-DM1, an antibody-cytotoxic drug conjugate. Cancer Res. 2008; 68(22): 9280-9290.
- Cronin KA, Harlan LC, Dodd KW, Abrams JS, Ballard-Barbash R. Population-based estimate of the prevalence of HER-2 positive breast cancer tumors for early stage patients in the US. Cancer Invest. 2010; 28(9): 963-968.
- Konecny G, Pauletti G, Pegram M, Untch M, Dandekar S, Aguilar Z, et al. Quantitative association between HER-2/neu and steroid hormone receptors in hormone receptor-positive primary breast

cancer. J Natl Cancer Inst. 2003; 95(2): 142-153.

- Huang HJ, Neven P, Drijkoningen M, Paridaens R, Wildiers H, Van Limbergen E, et al. Association between HER-2/neu and the progesterone receptor in oestrogen-dependent breast cancer is agerelated. Breast Cancer Res Treat. 2005; 91(1): 81-87.
- Ariga R, Zarif A, Korasick J, Reddy V, Siziopikou K, Gattuso P. Correlation of her-2/neu gene amplification with other prognostic and predictive factors in female breast carcinoma. Breast J. 2005; 11(4): 278-280.
- Sandhu GS, Erqou S, Patterson H, Mathew A. Prevalence of triplenegative breast cancer in India: Systematic review and meta-analysis. Journal of Global Oncology. 2016; 2(6): 412-421.
- Gutierrez C, Schiff R. HER2: biology, detection, and clinical implications. Arch Pathol Lab Med. 2011; 135(1): 55-62.
- Lambros MB, Natrajan R, Reis-Filho JS. Chromogenic and fluorescent in situ hybridization in breast cancer. Hum Pathol. 2007; 38(8): 1105-1122.
- Arafah M, Kfoury HK, Zaidi SN. HER2/neu immunostaining in invasive breast cancer: analysis of false positive factors. Oman Med J. 2010; 25(4): 261-263.
- Kostopoulou E, Vageli D, Kaisaridou D, Nakou M, Netsika M, Vladica N, et al. Comparative evaluation of non-informative HER-2 immunoreactions (2+) in breast carcinomas with FISH, CISH and QRT-PCR. Breast. 2007; 16(6): 615-624.
- McCormick SR, Lillemoe TJ, Beneke J, Schrauth J, Reinartz J. HER2 assessment by immunohistochemical analysis and fluorescence in situ hybridization: comparison of HercepTest and PathVysion commercial assays. Am J Clin Pathol. 2002; 117(6): 935-943.
- Rosa FE, Santos RM, Rogatto SR, Domingues MA. Chromogenic in situ hybridization compared with other approaches to evaluate HER2/neu status in breast carcinomas. Braz J Med Biol Res. 2013; 46(3): 207-216.
- Bahreini F, Soltanian AR, Mehdipour P. A meta-analysis on concordance between immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) to detect HER2 gene overexpression in breast cancer. Breast Cancer. 2015; 22(6): 615-625.
Transcription Levels of *nicotinamide nucleotide transhydrogenase* and Its Antisense in Breast Cancer Samples

Soraya Saleh Gargari, M.D.¹, Mohammad Taheri, M.Sc.², Vahid Kholghi Oskooei, Ph.D.³, Mir Davood Omrani, Ph.D.^{2, 3*}, Soudeh Ghafouri-Fard, M.D., Ph.D.^{3*}

Feto-Maternal Unit, Shohadaye Tajrish Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran
 Urogenital Stem Cell Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
 Department of Medical Genetics, Shahid Beheshti University of Medical Sciences, Tehran, Iran

*Corresponding Address: P.O.Box: 19857-17443, Department of Medical Genetics, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Emails: davood_omrani@yahoo.co.uk, s.ghafourifard@sbmu.ac.ir

Received: 21/July/2018, Accepted: 26/September/2018

Abstract

Objective: To evaluate association of patients' clinicopathological data with expression of nicotinamide nucleotide transhydrogenase (NNT) and naturally occurring antisense RNA of the same gene locus (NNT-AS1) in breast cancer samples.

Materials and Methods: In the current case-control study, mean expressions of *NNT* and *NNT-AS1* were assessed in 108 breast tissue samples including 54 invasive ductal carcinoma samples and 54 adjacent non-cancerous tissues (ANCTs) by quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

Results: *NNT* expression was not significantly different between tumor tissues and ANCTs. However, *NNT-AS1* expression was significantly down-regulated in tumor tissues compared to ANCTs (expression ratio=0.51, P=0.01). *NNT-AS1* expression was significantly higher in estrogen receptor (ER) negative samples, in comparison with ER positives (P=0.01). No considerable difference was found in the gene expressions between other subcategories of patients. Considerable correlations were detected between expression levels of these two genetic loci in both tumor tissues and ANCTs.

Conclusion: In the current study, for the first time we simultaneously assessed expression of *NNT* and *NNT-AS1* in breast cancer tissues. This study highlights association of ER status with dysregulation of *NNT-AS1* in breast cancer tissues. Future researches are necessary to explore the function of this long non-coding RNA (IncRNA) in the pathogenesis of breast cancer.

Keywords: Breast Cancer, Long Non-Coding RNA, NNT

Cell Journal(Yakhteh), Vol 21, No 3, October-December (Autumn) 2019, Pages: 331-336 _

Citation: Saleh Gargari S, Taheri M, Kholghi Oskooei V, Omrani MD, Ghafouri-Fard S. Transcription levels of nicotinamide nucleotide transhydrogenase and its antisense in breast cancer samples. Cell J. 2019; 21(3): 331-336. doi: 10.22074/cellj.2019.6238.

Introduction

Breast cancer, as the most frequent type of female cancer, has prompted several investigators to find diagnostic or prognostic biomarkers among long noncoding RNAs (lncRNAs) (1). This significant fraction of human transcriptome contributes to several aspects of cell physiology; so that dysregulation of them leads to development of cancer (2). An important subgroup of lncRNAs includes natural antisense transcripts (NATs) which are transcribed from the opposite DNA strand in relation to the sense transcripts, so their nucleotide sequences are complementary to the protein coding mRNA and the latter molecule is regulated by them. NATs can either inhibit or activate expression of the sense transcript (3).

Recent studies have highlighted contribution of *Nicotinamide nucleotide transhydrogenase (NNT)* and the related NAT in the pathogenesis of some human cancer types. NNT has an indispensible role in the homeostasis of NADH and NADPH (4). The

significant contribution of NNT in mitochondrial antioxidant pathways shields cells from oxidative stress (5). *NNT* silencing decreases ability of cancer cells to preserve NAD⁺ and NADPH levels and suppresses their proliferation and aggressive behavior possibly via alteration of HIF-1 α and HDAC1-dependent pathways (4). The related antisense transcript (*NNT-AS1*) participates in proliferation, migration, invasion and metastasis of colorectal cancer (CRC) cells (6). In hepatocellular carcinoma (HCC), *NNT-AS1* expression has enhanced cell proliferation and inhibited cycle arrest as well as apoptosis through modulation of *miR-363*/CDK6 axis (7).

NNT loss has led to accumulation of acetylated catabolic substances of polyamines and a subsequent diminution of spermine and spermidine. Polyamine catabolism would mutually be elicited by oxidative stress and over-produce hydrogen peroxide, resulting in a malicious cycle that accelerates reactive oxygen species (ROS) production (8, 9). In spite of the

appreciated role of these processes in pathogenesis of breast cancer (8), no study has yet assessed the simultaneous significance of *NNT* and *NNT-AS1* dysregulation in breast cancer. Consequently, we conducted current study to assess expression of *NNT* and *NNT-AS1* in breast cancer tissues compared to ANCTs in association with patients' clinicopathological data, to find whether their transcript levels are altered in breast cancer in parallel, or they can be used as biomarkers of breast cancer.

Material and Methods

Patients

For the current case-control study, a total of 108 breast tissue samples -including 54 invasive ductal carcinoma samples and 54 ANCTs- were excised during surgery from patients hospitalized in Farmanieh and Sina Hospitals (Tehran, Iran) during January 2017-January 2018. Patients with definite diagnosis of invasive ductal carcinoma were included in the study. Those with other types of breast cancer and those received chemo-/radio-therapy before surgery were excluded from the study. All patients signed the written informed consent forms. The study protocol was permitted by the Ethical Committee of Shahid Beheshti University of Medical Sciences (IR.REC. SBMU.1397.764), Iran. Clinical and demographical data of patients were collected through evaluation of medical reports and interviews with patients.

Expression analysis

Total RNA was extracted from tumor tissues and ANCTs using the TRIzol[™] reagent (Invitrogen, USA) based on the company guidelines. In brief, we homogenized 75 mg of tissues in 1 ml TRIzol[™] reagent, followed by RNA precipitation using isopropanol and washing it in 75% ethanol (10). After assessment of quality and quantity of the isolated total RNAs, a proportion of each RNA sample was converted to cDNA, using the RevertAid First Strand cDNA Synthesis Kit (TaKaRa, Japan). Transcript levels of NNT and NNT-AS1 genes were compared between tumor tissues and ANCTs using rotor gene 6000 Real-Time PCR System (Corbett Research, Australia). TagMan Fast Universal PCR Master Mix (Applied Biosystems, USA) was used for the expression study. The Hypoxanthineguanine phosphoribosyl transferase (HPRT) gene was used for normalization of the gene and lncRNA expressions. The nucleotide sequences of primers are as follow:

HPRT1-

F: 5'-AGCCTAAGATGAGAGTTC-3', R: 5'-CACAGAACTAGAACATTGATA-3', FAM-CATCTGGAGTCCTATTGACATCGC-TAMRA;

```
NNT1-
F: 5'-AGCCACCTTCTGTGTTACTTGC-3',
R: 5'-TAGCCCAGAGCTGCCATGAC-3',
FAM-TCAACCGTCAGGCTGCCACTGCTG-TAMRA
```

NNT-AS1-

```
F: 5'-CTTCCACTCTCGGGGGACAGG-3',
R: 5'-GCACCAGGTTTGATTGACAAGG-3',
FAM-TTGTCTCTGCCTCGGCCTGCGG-TAMRA.
```

PCR efficiency and threshold cycle (Ct) values were obtained to quantify relative expression of each genetic locus in tumor tissues and ANCTs.

Statistical analysis

SPSS software version 18.0 (SPSS Inc., USA) was used for statistical analysis. Ct values obtained from qRT-PCR experiments were adjusted based on the PCR efficiency values. Association of patients' data with relative expression of the gene or lncRNA (down-/ up-regulation in the tumor samples vs. ANCTs) was evaluated using Chi-square test. Relative expression of each genetic loci in each tumor sample was calculated using Efficiency^{Ct reference}/ Efficiency^{Ct target} formula. Data is presented as mean \pm SD. The difference in these values between individual groups of patients was evaluated using Tukey's honest significance test. The pairwise correlation between relative transcription levels of the genetic loci in each set of samples (tumor tissues and ANCTs) was calculated using the regression model. For all statistical analyses, P<0.05 was regarded as significant. The receiver operating characteristic (ROC) curve was plotted to assess the power of genetic loci expression levels for diagnosis of disease status in breast samples. The Youden index (i) was applied to get the highest difference between sensitivity (true-positive rate) and 1-specificity (falsepositive rate).

Results

Overall demographic and clinical information of patients

Demographic and clinical information of the study participants are reported in Table 1.

Transcript levels of *NNT* and *NNT-AS1* in tumor tissues and ANCTs

NNT expression level was not significantly different between tumor tissues and ANCTs. However, *NNT-AS1* expression was significantly down-regulated in tumor tissues compared to ANCTs (expression ratio=0.51, P=0.01). Figure 1 shows relative expression of *NNT* and *NNT-AS1* in each set of samples, as described by - Δ Ct values (Ct_{reference}-Ct_{tareet}).

Table 1: General demographic and clinical data of pa	atients
--	---------

Variables	Values
Age (Y)	51.79 ± 13.54 (29-81)
Menarche age (Y)	13 ± 1.65 (10-18)
Menopause age (Y)	44.91 ± 14.91 (38-60)
First pregnancy age (Y)	18.04 ± 8.36 (14-32)
Breast feeding duration (months)	41.62 ± 34.1 (3-120)
Positive family history for other cancers	17
Cancer stage	
Ι	30.8
П	28.8
III	30.8
IV	9.6
Overall grade	
Ι	17
П	49
III	34
Mitotic rate	
Ι	45.2
П	42.9
III	11.9
Tumor size	
<2 cm	32
≥2 cm, <5cm	66
≥5 cm	2
Estrogen receptor	
Positive	87.8
Negative	12.2
Progesterone receptor	
Positive	77.1
Negative	22.9
HER2/Neu expression	
Positive	25
Negative	75



Fig.1: Relative transcription of NNT and NNT-AS1 in tumor tissues (n=54) and ANCTs (n=54).

NNT; Nicotinamide nucleotide transhydrogenase, NNT-AS1; Nicotinamide nucleotide transhydrogenaseantisense 1, ANCTs; Adjacent non-cancerous tissues, and ct; Threshold cycle.

Association between relative expression of genetic loci and patients' clinicopathological information

Based on the transcriptions in each tumor sample compared to related ANCT (<1 or >1), we categorized patients to up-/down-regulated groups. Then, we evaluated associations between such values and patients' clinicopathological data. No association was found between relative expression of genetic loci in tumor tissue vs. ANCT and any of tumor characteristics (Table 2).

We also calculated relative expression of each genetic loci in tumor samples using Efficiency^{Ct reference/} Efficiency^{Ct target} formula and compared these values between tumor subgroups. We detected significantly higher expression of *NNT-AS1* in ER negative samples compared to ER positive cases (P=0.01). No remarkable difference was found between transcription levels of the other patient subcategories (Table 3).

Correlations between transcript levels of *NNT* and *NNT-AS1* in tumor tissues and ANCTs

Correlations between transcript level of *NNT* and its naturally occurring antisense was assessed in both tumor and ANCT samples. Transcription levels of *NNT* were correlated with the expression of *NNT*-*AS1* in both ANCT and tumor samples (Fig.2A, B, respectively). Considering R² values, the correlation was stronger in ANCTs compared to tumor tissues.

Data are presented as mean ± SD (range) or %.

NNT-AS1 Expression in Breast Cancer

Paramotors	NNT	NNT	Pyalua			P voluo
Parameters	Up-regulation	Down-regulation	P value	NN1-AS1 Up-regulation	NNI-ASI Down-regulation	P value
Age			0.4			0.79
<55 Y	15 (45.5)	18 (54.5)		12 (35.3)	22 (64.7)	
≥55 Y	6 (33.3)	12 (66.7)		7 (38.9)	11 (61.1)	
Stage			0.9			0.76
Ι	6 (37.5)	10 (64.5)		5 (33.3)	10 (66.7)	
II	5 (35.7)	9 (64.3)		7 (46.7)	8 (53.3)	
III	7 (46.7)	8 (53.3)		5 (56.3)	11 (43.7)	
IV	2 (50)	2 (50)		1 (25)	3 (75)	
Histological grade			0.38			0.97
I	5 (62.5)	3 (37.5)		3 (37.5)	5 (62.5)	
П	8 (34.8)	15 (65.2)		8 (36.4)	14 (63.6)	
III	5 (37.5)	8 (62.5)		5 (33.3)	10 (66.7)	
Mitotic rate			0.57			0.26
Ι	8 (42.1)	11 (57.9)		9 (50)	9 (50)	
П	5 (33.3)	10 (66.7)		4 (23.5)	13 (76.5)	
III	3 (60)	2 (40)		2 (40)	3 (60)	
Tumor size			0.67			0.31
<2	7 (43.8)	9 (56.2)		7 (43.8)	9 (56.2)	
2-5	12 (38.7)	19 (61.3)		10 (32.3)	21 (67.7)	
>5	0 (0)	1 (100)		1 (100)	0 (0)	
ER status			0.84			0.33
Positive	17 (40.5)	21 (58.3)		15 (36.6)	26 (63.4)	
Negative	2 (40)	3 (60)		1 (16.7)	5 (83.3)	
PR status			0.92			0.54
Positive	15 (41.7)	21 (58.3)		13 (37.1)	22 (62.9)	
Negative	4 (40)	6 (60)		3 (27.3)	8 (72.7)	
HER2 status			0.51			0.54
Positive	4 (33.3)	8 (66.7)		3 (27.3)	8 (72.7)	
Negative	15 (44.1)	19 (54.3)		13 (37.1)	22 (62.9)	

NNT; Nicotinamide nucleotide transhydrogenase, NNT-AS1; Nicotinamide nucleotide transhydrogenase-antisense 1, ANCTs; Adjacent noncancerous tissues, ER; Estrogen receptor, PR; Progesterone receptor, and HER2; Human epidermal growth factor receptor 2.

 Table 3: Association of transcription levels in tumor tissues with tumors characteristics (mean ± SD values of Efficiency^{Ct reference} - Efficiency^{Ct target} arepresented)

Parameters	NNT	P value	NNT-AS1	P value
Age				
<55 vs. ≥55 Y	71.97 (221.19) vs. 2.6 (4.63)	0.18	33.96 (127.22) vs. 2.79 (4.94)	0.29
ER status				
ER(+) vs. ER(-)	54.76 (198.15) vs. 20.84 (45.58)	0.68	10.58 (39.1) vs. 119.63 (289.12)	0.01
PR status				
PR(+) vs. PR(-)	63.53 (212.69) vs. 11.72 (33.89)	0.42	12.23 (42) vs. 57.81 (213.71)	0.15
HER2 status				
HER2 (+) vs. HER2 (-)	1.17 (2.78) vs. 68.49 (215.27)	0.28	1.53 (4.68) vs. 32.06 (123.7)	0.4
Tumor grade				
Grade I vs. II	84.71 (222.77) vs. 28.58 (131.89)	0.76	5.61 (7.02) vs. 13.89 (52.76)	0.98
Grade I vs. III	84.71 (222.77) vs. 75.51 (253.41)	0.99	5.61 (7.02) vs. 53.38 (182.01)	0.58
Grade II vs. III	28.58 (131.89) vs. 75.51 (253.41)	0.74	13.89 (52.76) vs. 53.38 (182.01)	0.53

NNT; Nicotinamide nucleotide transhydrogenase, NNT-AS1; Nicotinamide nucleotide transhydrogenase-antisense 1, ct; Threshold cycle, ER; Estrogen receptor, PR; Progesterone receptor, and HER2; Human epidermal growth factor receptor 2. Data are presented as mean (SD) values.



Fig.2: Correlation of *NNT* and *NNT-AS1* transcription levels. **A.** Shows the correlation in ANCTs and **B.** Shows the correlations in tumor tissues. *NNT; Nicotinamide nucleotide transhydrogenase, NNT-AS1; Nicotinamide nucleotide transhydrogenase- antisense 1,* and ANCTs; Adjacent non-cancerous tissues.

Receiver operating characteristic curve analysis

The power of *NNT-AS1* expression in prediction of disease status in breast samples was evaluated using ROC curve (Fig.3). Assessment of this lncRNA transcription level shows 71.2% specificity and 56.6% sensitivity for breast cancer diagnosis.



Fig.3: Results of receiver operating characteristic (ROC) curve assessment analysis for *NNT-AS1* performance in breast cancer diagnosis. The values were analysed based on Efficiency^{Ct reference} - Efficiency^{Ct target}.

Discussion

In the current study, for the first time we simultaneously assessed expression of NNT and NNT-AS1 in breast cancer tissues in comparison with ANCTs and found downregulation of NNT-AS1 in tumor tissues, in spite of detecting similar level of NNT expression in tumor tissues and ANCTs. *NNT-ASI* has previously been shown to exert oncogenic effects in CRC, HCC, osteosarcoma and cervical cancer (6, 7, 11, 12). However, expression of this lncRNA was significantly decreased in patients with ovarian cancer as well as the human ovarian cancer cell lines. Moreover, in vitro studies has shown that NNT-AS1 silencing enhances cell migration and invasion, while it suppresses apoptosis (13). So, the observed down-regulation of *NNT-AS1* in the current study is consistent with the previously reported dysregulation of this molecule in ovarian cancer. This lncRNA is transcribed in the opposite direction of NNT gene and it has no intersection with latter gene nucleotide sequence (14). Most recently, Li et al. (14) demonstrated overexpression of NNT-AS1 in breast tumor tissues compared to ANCTs in correlation with patients' survival and HER2, but not ER, status. In vitro experiments showed that NNT-ASI contributes to breast cancer pathogenesis via altering miR-142-3p/ZEB1 axis. The inconsistency between our results and their results might be due to a possible difference in the mean age of the study participants or an ethnic-based modulator of transcription. They have shown that ZEB1 is positively regulated by NNT-AS1. In our previous study, on the same cohort of patients, we failed to demonstrate any significant change in ZEB1 expression between tumor tissues and ANCTs (15). So, we hypothesized that NNT-ASI might participate in the pathogenesis of breast cancer through other mechanisms, including regulation of NNT expression. Significant correlation between NNT and NNT-ASI expressions, especially in non-tumor tissues, implies the presence of a feed-forward loop between these two genetic loci which should be assessed in future studies.

In spite of totally down-regulation of *NNT-AS1* in tumor tissues compared to ANCTs, we demonstrated higher expression of it in ER negative tumor samples, compared to ER positive samples, likely suggesting the importance of this lncRNA in pathogenesis of ER negative breast cancers. Future studies are needed to evaluate expression of *NNT-AS1* in larger cohorts of patients with regards to hormone receptor status.

Finally, we evaluated the power of *NNT-AS1* expression in prediction of the disease status in breast samples. Although transcription level of this genetic locus is not individually a sensitive marker for prediction of breast cancer, it might increase specificity of other putative panels of gene expression.

Conclusion

The current study shows down-regulation of *NNT-AS1* in breast cancer tissues compared to ANCTs in association with ER status. Future studies are necessary to explore function of this lncRNA in the pathogenesis of breast cancer.

Acknowledgments

The current study was financially supported by Shahid Beheshti University of Medical Sciences (grant number: 15033), Iran. There is no conflict of interest in this study.

Authors' Contributions

S.S.G., V.K.O., M.D.O, S.G.-F; Contributed to conception and design. M.T.; Contributed to all experimental work, data and statistical analysis, as well as interpretation of data. M.D.O., S.G.-F.; Supervised the experiments. S.G.-F.; Drafted the manuscript, which was revised by M.D.O. and S.S.G. All authors read and approved the final manuscript.

References

- Tasharrofi B, Soudyab M, Nikpayam E, Iranpour M, Mirfakhraie R, Sarrafzadeh S, et al. Comparative expression analysis of hypoxiainducible factor-alpha and its natural occurring antisense in breast cancer tissues and adjacent noncancerous tissues. Cell Biochem Funct. 2016; 34(8): 572-578.
- Nikpayam E, Tasharrofi B, Sarrafzadeh S, Ghafouri-Fard S. The role of long non-coding RNAs in ovarian cancer. Iran Biomed J. 2017; 21(1): 3-15.
- Fang Y, Fullwood MJ. Roles, Functions, and mechanisms of long non-coding RNAs in cancer. Genomics Proteomics Bioinformatics. 2016; 14(1): 42-54.
- Ho HY, Lin YT, Lin G, Wu PR, Cheng ML. Nicotinamide nucleotide transhydrogenase (NNT) deficiency dysregulates mitochondrial retrograde signaling and impedes proliferation. Redox Biol. 2017; 12: 916-928.
- Chortis V, Taylor AE, Doig CL, Walsh MD, Meimaridou E, Jenkinson C, et al. Nicotinamide Nucleotide Transhydrogenase as a novel treatment target in adrenocortical carcinoma. Endocrinology. 2018; 159(8): 2836-2849.
- Wang Q, Yang L, Hu X, Jiang Y, Hu Y, Liu Z, et al. Upregulated NNT-AS1, a long noncoding RNA, contributes to proliferation and migration of colorectal cancer cells in vitro and in vivo. Oncotarget. 2017; 8(2): 3441-3453.
- Lu YB, Jiang Q, Yang MY, Zhou JX, Zhang Q. Long noncoding RNA NNT-AS1 promotes hepatocellular carcinoma progression and metastasis through miR-363/CDK6 axis. Oncotarget. 2017; 8(51): 88804-88814.
- Pirinen E, Kuulasmaa T, Pietilä M, Heikkinen S, Tusa M, Itkonen P, et al. Enhanced polyamine catabolism alters homeostatic control of white adipose tissue mass, energy expenditure, and glucose metabolism. Mol Cell Biol. 2007; 27(13): 4953-4967.
- Chopra S, Wallace HM. Hydrogen peroxide induces the catabolism of polyamines in human breast cancer cells. Biochem Soc Trans. 1996; 24(2): 230S.
- Rio DC, Ares M Jr, Hannon GJ, Nilsen TW. Purification of RNA using TRIzol (TRI reagent). Cold Spring Harb Protoc. 2010; 2010(6): pdb.prot5439.
- Hua F, Liu S, Zhu L, Ma N, Jiang S, Yang J. Highly expressed long non-coding RNA NNT-AS1 promotes cell proliferation and invasion through Wnt/beta-catenin signaling pathway in cervical cancer. Biomed Pharmacother. 2017; 92: 1128-1134.
- Ye H, Lin J, Yao X, Li Y, Lin X, Lu H. Overexpression of Long Non-Coding RNA NNT-AS1 Correlates with Tumor Progression and Poor Prognosis in Osteosarcoma. Cell Physiol Biochem. 2018; 45(5): 1904-1914.
- Huang Y, Shi J, Xu Y. Long non-coding RNA NNT-AS1 contributes to cell proliferation, metastasis and apoptosis in human ovarian cancer. Oncol Lett. 2018; 15(6): 9264-9270.
- Li Y, Lv M, Song Z, Lou Z, Wang R, Zhuang M. Long non-coding RNA NNT-AS1 affects progression of breast cancer through miR-142-3p/ZEB1 axis. Biomed Pharmacother. 2018; 103: 939-946.
- Nikpayam E, Soudyab M, Tasharrofi B, Sarrafzadeh S, Iranpour M, Geranpayeh L, et al. Expression analysis of long non-coding ATB and its putative target in breast cancer. Breast Dis. 2017; 37(1): 11-20.

Investigation of Chromosomal Abnormalities and Microdeletion/ Microduplication(s) in Fifty Iranian Patients with Multiple **Congenital Anomalies**

Akbar Mohammadzadeh, M.D.¹, Susan Akbaroghli, M.D.^{2, 3}, Ehsan Aghaei-Moghadam, M.D.⁴, Nejat Mahdieh, Ph.D.⁵, Reza Shervin Badv, M.D.⁴, Payman Jamali, M.D.⁶, Roxana Kariminejad, Ph.D.⁷, Zahra Chavoshzadeh, M.D.^{8, 9}, Saghar Ghasemi Firouzabadi, Ph.D.¹, Roxana Mansour Ghanaie, M.D.⁹, Ahoura Nozari, Ph.D.¹, Sussan Banihashemi, B.Sc.¹, Fatemeh Hadipour, M.D.^{10, 11}, Zahra Hadipour, M.D.^{10, 11}, Ariana Kariminejad, M.D.⁷, Hossein Najmabadi, Ph.D.^{1, 7}, Yousef Shafeghati, M.D.^{10, 11}, Farkhondeh Behjati, Ph.D.^{1, 10, 11}*

1. Genetics Research Center, University of Social Welfare and Rehabilitation Sciences, Tehran, Iran 2. Pediatric Neurology Research Center, Mofid Children's Hospital, Faculty of Medicine, Shahid Beheshti University of Medical Sciences,

Pediatric Neurology Research Center, Mond Crimer's Hospital, Paculty of Medicine, Shahid Benesiti Oniversity of Medical Sciences, Tehran, Iran
 Clinical Genetics Division, Mofid Children's Hospital, Faculty of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
 Children's Medical Center, Pediatrics Center of Excellence, Tehran University of Medical Sciences, Tehran, Iran
 Cardiogenetic Research Laboratory, Rajaie Cardiovascular Medical and Research Center, Iran University of Medical Sciences,

Tehran, Iran 6. Genetic Counseling Center, Shahroud Welfare Organization, Shahroud, Iran 7. Kariminejad-Najmabadi Pathology and Genetics Center, Tehran, Iran 8. Department of Immunology and Allergy, Mofid Children's Hospital, Faculty of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

9. Pediatric Infections Research Center, Research Institute for Children's Health, Faculty of Medicine, Shahid Beheshti University of

Medical Sciences, Tehran, Iran 10. Sarem Fertility and Infertility Research Center (SAFIR), Sarem Women's Hospital, Tehran, Iran

11. Sarem Cell Research Center (SCRC), Sarem Women's Hospital, Tehran, Iran

*Corresponding Address: P.O.Box: 1985713871, Genetics Research Center, University of Social Welfare and Rehabilitation Sciences, Daneshjoo Blvd, Koodakyar St, Evin, Tehran, Iran

Email: f behjati@uswr.ac.ir

Received: 30/May/2018, Accepted: 21/October/2018 Abstract

Objective: Major birth defects are inborn structural or functional anomalies with long-term disability and adverse impacts on individuals, families, health-care systems, and societies. Approximately 20% of birth defects are due to chromosomal and genetic conditions. Inspired by the fact that neonatal deaths are caused by birth defects in about 20 and 10% of cases in Iran and worldwide respectively, we conducted the present study to unravel the role of chromosome abnormalities, including microdeletion/microduplication(s), in multiple congenital abnormalities in a number of Iranian patients.

Materials and Methods: In this descriptive cross-sectional study, 50 sporadic patients with Multiple Congenital Anomalies (MCA) were selected. The techniques employed included conventional karyotyping, fluorescence in situ hybridization (FISH), multiplex ligation-dependent probe amplification (MLPA), and array comparative genomic hybridisation (array-CGH), according to the clinical diagnosis for each patient.

Results: Chromosomal abnormalities and microdeletion/microduplication(s) were observed in eight out of fifty patients (16%). The abnormalities proved to result from the imbalances in chromosomes 1, 3, 12, and 18 in four of the patients. However, the other four patients were diagnosed to suffer from the known microdeletions of 22q11.21, 16p13.3, 5q35.3, and 7q11.23.

Conclusion: In the present study, we report a patient with 46,XY, der(18)[12]/46,XY, der(18), +mar[8] dn presented with MCA associated with hypogammaglobulinemia. Given the patient's seemingly rare and highly complex chromosomal abnormality and the lack of any concise mechanism presented in the literature to justify the case, we hereby propose a novel mechanism for the formation of both derivative and ring chromosome 18. In addition, we introduce a new 12q abnormality and a novel association of an Xp22.33 duplication with 1q43q44 deletion syndrome. The phenotype analysis of the patients with chromosome abnormality would be beneficial for further phenotype-genotype correlation studies.

Keywords: Array Comparative Genomic Hybridization, Chromosomal Abnormalities, Congenital Abnormalities, Microdeletions, Multiplex Ligation-Dependent Probe Amplification

Cell Journal(Yakhteh), Vol 21, No 3, October-December (Autumn) 2019, Pages: 337-349

Citation: Mohammadzadeh A, Akbaroghli S, Aghaei-Moghadam E, Mahdieh N, Badv RS, Jamali P, Kariminejad R, Chavoshzadeh Z, Ghasemi Firouzabadi S. Mansour Ghanaie R. Nozari A. Banihashemi S. Hadipour F. Hadipour Z. Karimineiad A. Naimabadi H. Shafeqhati Y. Behiati F. Investigation of chromosomal abnormalities and microdeletion/microduplication(s) in fifty Iranian patients with multiple congenital anomalies. Cell J. 2019; 21(3): 337-349. doi: 10.22074/ cellj.2019.6053.

Introduction

Major birth defects are considered to be inborn structural or functional abnormalities which could be diagnosed prenatally, at birth, or later in infancy, or even in adulthood. Their consequence will be

a long-term disability with major adverse effects on individuals, families, health-care systems, and societies (1). Approximately 20% and more than 10% of all neonatal deaths in Iran and worldwide (2) are caused by birth defects, respectively. The incidence of major birth defects in neonatals, resulting from genetic or partially genetic factors, is about 7.9 million per year (3). A fifth (20.2%) of the cases with birth defects are attributed to a known etiology comprising of chromosomal (15.8%) and genetic (3.8%) conditions. Teratogens are the cause of 0.8% of cases, 0.3% of them are caused by twinning, and four-fifths (79.8%) are categorized to be of unknown etiology (1). Nearly, 20 to 30% of the infants suffering from birth defects have multiple congenital anomalies (MCA) involving different organs. In cases where two or more major congenital abnormalities occur in several organs and the defects do not represent a sequence or a complex series, the case is classified as MCA (4).

Genetic factors are regarded as one of the most prominent etiologies of MCA (1). Also, chromosomal abnormalities have been known to be one of the leading causes of intellectual disability (ID) as well as congenital malformations. A large number of chromosomal defects can be detected immediately through high-resolution karyotyping technique. But, the resolution of the cytogenetic analysis is restricted to around 5 to 10 Mb. Among molecular cytogenetic techniques, fluorescence in situ hybridization (FISH) and multiplex ligation-dependent probe amplification (MLPA) are two of the targeted tests used to detect submicroscopic chromosome abnormalities. Genomewide molecular cytogenetic tests like array comparative genomic hybridisation (array-CGH) can identify a variety of copy number variants (CNVs) associated with MCA. Array-CGH, as a powerful test, is applied to investigate individuals with MCA, ID, and autism spectrum disorders (5). In the current study, we aimed at identifying the chromosomal abnormalities and microdeletion/microduplication(s) in 50 Iranian patients with MCA. The patients manifested diverse phenotypes including ID/developmental delay (DD), and at least one major congenital anomaly in another organ. Existence of additional minor dysmorphic features was also considered among the factors representing chromosomal abnormalities which led to an increase in the detection rate.

Materials and Methods

Selection of patients

In this descriptive cross-sectional study conducted during the three past years from April 2015 to May 2018, clinical evaluation was performed for 50 selected sporadic patients suffering from MCA preferably born to unrelated parents and referred to us by experienced clinical specialists for genetic investigation from all over the country. All of the patients had ID/DD with at least one major anomaly and additional minor dysmorphic features. All of the steps taken for testing and procedures were fully explained for all of the probands' parents, and signed informed consent forms from all of the participants were obtained for publishing any information or accompanying photographs. This study was approved by the Ethics Committee of the University of Social Welfare and Rehabilitation Sciences (IR.USWR.REC.1394.186).

Conventional karyotyping

A conventional cytogenetic study was carried out on peripheral blood lymphocytes using GTG high-resolution banding technique according to standard protocols for all patients (6). Twenty GTG banded metaphases were examined through the complete analysis of each individual sample. Chromosome analysis was performed according to ISCN 2016 (7).

Fluorescence in situ hybridization

Metaphase FISH was done only for patient 9 using the centromeric probe of chromosome 18 (Kreatech/ Leica Biosystems Buffalo Grove, IL, USA, http://www. leicabiosystems.com) according to Kreatech protocol (8). Two hundred cells were examined to characterize the ring chromosome origin defined in 37% of cells by highresolution GTG banding.

Multiplex ligation-dependent probe amplification

MLPA technique was done using P245 Microdeletion Syndromes-1 kit (MRC-Holland, Amsterdam, the Netherlands) for the patients suspected of microdeletion/ microduplication syndromes. MLPA protocol was performed based on the instructions provided by the manufacturer, MRC Holland, and the data were analyzed utilizing capillary electrophoresis in the 3130XL DNA Analyzer, Coffalyser. Net software (MRC-Holland, Amsterdam, the Netherlands) and Gene Marker software version 2.7.0 (Softgenetics, State College, PA, USA). Abnormal results obtained with MLPA assay were repeated and if different, further investigations were carried out with array-CGH. MLPA studies were done for the patients' parents with abnormal results in order to determine the causal role (de novo or inherited) of microdeletions.

Array comparative genomic hybridization

The analysis of oligonucleotide array-CGH for Genomic DNA was carried out. To do so, the BlueGnome CytoChip ISCA 8×60 K v2.0 wholegenome oligo array was utilized for patients 1, 5, 9, 13, and 41. This array included intragenic and intergenic probe spacings of about 48 kb and 70 kb, respectively and high probe density in 500 clinically important regions. CytoSNP-850K v1.1 BeadChip overall effective resolution of about 18 Kb was used for patient 27. The tests were performed based on the manufacturer's protocol. INNOPSYS 910 laser scanner was utilized for scanning according to the recommended protocol of the manufacturer. Image analysis and base calling were carried out employing the BlueFuse Multi-version 3 analysis software for oligo array and Multi-version 4.4 analysis software

for SNP array. The analysis of the samples was carried out two times against two non-identical controls and representation of minimum three clones on the platform in both of the experiments was considered to approve the imbalances. To evaluate the called CNVs, we utilized the standards and guidelines of American College of Medical Genetics and Genomics to interpret the postnatal constitutional CNVs (5). All chromosome coordinates are on the basis of GRCh37. p13/hg19.

Results

Based on the selection criteria, the patients had MCA, i.e., having ID/DD and at least one major congenital anomaly in another organ plus additional minor facial dysmorphic features. The clinical characteristic features of all patients with MCA are summarized in Table 1. The participants included 25 male and 25 female patients. Their ages ranged from 7 months to 21 years with a median age of 3.25 years and an average of about 5.1 years (Table 1).

Excluding ID/DD, the most common clinical features from the highest frequent to the lowest frequent were congenital heart defects in 45 patients (90%), craniofacial dysmorphic features in 31 patients (62%), musculoskeletal, ear and genitourinary abnormalities, each one in 19 patients (38%), eye abnormalities in 16 patients (32%), gastrointestinal abnormalities in 5 patients (10%), and endocrine abnormalities in 3 patients (6%) (Table 2).

In patient 9, a 31-month-old boy from consanguineous parents (half first cousins) and born to a 27-year-old G1P1L1A0 (G: gravid, P: parity, L: live birth, A: abortus) mother, the clinical features were global DD, PDA, PFO, optic nerve hypoplasia, hypogammaglobulinemia, facial dysmorphic features, microcephaly, and mild ventriculomegaly in frontal horns in the axial spiral CT-scan of the brain without contrast, and benign extra-axial hydrocephalus and atrophic changes in the brain magnetic resonance imaging (MRI). The karyotype analysis by highresolution GTG banding showed 46,XY, der(18) [12]/46,XY, der(18), +mar[8] dn (Fig.1A1, 2). Since in both cell lines of the patient the short arm of one chromosome 18 (18p) was abnormal and one cell line had an additional ring chromosome, one hundred cells were screened to obtain the percentage of the cells with marker ring chromosome. The karvotype analyses of the proband's parents were normal. Metaphase FISH using the centromeric probe for chromosome 18 indicated three hybridization signals for chromosome 18 in 37% of the cells scored which was consistent with mosaic trisomy 18, nuc ish $(D18Z1\times3)[37]/(D18Z1\times2)$ [63] dn (Fig.1A3, 4). Further investigations using array-CGH demonstrated the exact breakpoints of the derivative chromosome 18 and its deletion and duplication (Fig.1A5).



Fig1: Results of cytogenetic/molecular cytogenetic analysis of patients 9, 17, and 41. A1. Abnormal G-banded chromosomes 18 observed in all spreads, A2. 37% of spreads included ring 18, der(18) and normal 18 from left to right, A3, A4. FISH results using centromeric probe for chromosome 18 (arrowed), A5. Array-CGH profile of chromosome 18 utilizing the BlueGnome CytoChip ISCA 8x60K v2.0 whole genome oligo array, B1. G-banded der(4), B2. Balanced translocation of chromosomes 4 and 12, and C. G-banded chromosomes 3 with 3p25.3p26.3 deletion. The normal idiograms of the mentioned chromosomes are in accordance with ISCN 2016 (7).

	Table 1: The clinical features of 50 patients with MCA								
Patient	Sex	Age	Clinical findings						
1	F	14 month	DD, SVAS, SVPS, IUGR, Hypotonia, Left visual defect, Facial dysmorphic features, Strabismus, ADHD, Dental problems, Congenital hypothyroidism						
2	М	25 month	DD, VSD, ASD, Pulmonary hypertension, Microcephaly, Facial dysmorphic features, UDT, Small testes						
3	F	8 Y	ID, TOF, PDA, Microcephaly, DD, Aggressiveness, Umbilical hernia, Flat feet, Syndactyly						
4	М	3.5 Y	DD, ASD, PDA, Bilateral congenital glaucoma, Hypotonia, Bilateral club feet, Bilateral hip dislocation (grade 4), Bilateral hydronephrosis, Bilateral inguinal hernia and hydrocele, Hypothyroidism						
5	F	5.5 Y	ID, Large ASD, Epilepsy, Autistic behavior, Macrocephaly, Dolicocephaly, Facial dysmorphic features, Sparse hair, Joint laxity, Severe left renal reflux						
6	F	6 Y	ID, VSD, ASD, Microcephaly, Bilateral club feet, Joint stiffness						
7	М	2.5 Y	DD, Large ASD, Bilateral SNHL, Bilateral hydronephrosis, Dysphagia, Hypotonia, Polydactyly						
8	М	3.5 Y	DD, TR, Facial paralysis, Left SNHL						
9	М	31 month	Global DD, PDA, PFO, Hypotonia, Optic nerve hypoplasia, Facial dysmorphic features, Recurrent lower respiratory tract infection, Hypogammaglobulinemia, Dysphagia, Microcephaly, Speech disorder, Hypertelorism, Micrognathia, Clinodactyly, Rocker bottom feet, Short stature, Fecal/urinary incontinence						
10	М	4 Y	DD, Severe AS, Severe PS, ASD, Facial dysmorphic features, Brachydactyly, Clinodactyly, Flat feet, Hirsutism						
11	F	4 Y	DD, TOF, IUGR, Hypotonia, ADHD, Insomnia, Microcephaly, Facial dysmorphic features, Brachydactyly, Bilateral simian creases, Low-set ears						
12	М	1.5 Y	DD, TOF, Hypotonia, Facial dysmorphic features, Low-set ears, Bilateral UDT						
13	М	3 Y	DD, VSD, AR, Bilateral SNHL, Facial dysmorphic features, Bilateral UDT, Speech delay, Malformed, Posteriorly rotated and low set ears, Simian crease, 5 th toe clinodactyly, Umbilical hernia, Sacral mongolian spot, Right lower limb paresis, Joint contractures, Bilateral pachygyria						
14	М	4.5 Y	DD, TOF, Hypotonia, Bilateral SNHL, Urinary reflux						
15	М	3 Y	DD, Large ASD, Hypotonia, Microcephaly, Facial dysmorphic features, High-arched palate						
16	F	3 Y	DD, ASD, Hypotonia, Facial dysmorphic features, Speech delay, Macrocephaly, Wide anterior fontanel						
17	М	8.5 month	ID, DD, PDA, ASD, Hypotonia, Facial dysmorphic features, Infantile spasms and myoclonic jerks, Hearing loss, Bilateral simian creases, Corpus callosum hypoplasia, Growth retardation, Abnormal EEG, Microcephaly, Short neck, Malformed ears, Low-set ears, High-arched palate, Camptodactyly, Joint hyperlaxity, Imperforated anus, Umbilical hernia, Bilateral UDT, Small kidneys						
18	М	5.5 Y	ID, Large ASD, PS, PDA, Hypotonia, Long philtrum, Visual defect, Bilateral inguinal hernia						
19	F	3.5 Y	DD, Severe PS, Facial dysmorphic features						
20	F	3 Y	DD, TOF, Bilateral congenital anophthalmia, Absence of manubrium						
21	F	2 Y	DD, ASD, Bilateral SNHL, Facial dysmorphic features, Right periauricular and periorbital tags, Rectovaginal fistula, Right lower lid coloboma						
22	М	7 Y	ID, VSD, Hypotonia, Corpus callosum hypoplasia, DD, Facial dysmorphic features						
23	М	2.5 Y	DD, TGA, Bilateral SNHL, Strabismus, High-arched palate, Clinodactyly						
24	М	2 Y	DD, Large VSD, PDA, Pulmonary hypertension, Left SNHL, Microcephaly, Bilateral UDT, Ambiguous genitalia						
25	F	5.5 Y	ID, Large VSD, Pulmonary hypertension, Hypotonia, Visual defect, Epilepsy, Bilateral simian creases, Microcephaly, Hyperreflexia						
26	М	2 Y	DD, TOF, Hypotonia, Facial dysmorphic features						

Table 1: Continued									
Patient	Sex	Age	Clinical findings						
27	М	18 Y	ID, TOF, DD, Microcephaly, Hypotonia, Severe scoliosis, Short stature, Facial dysmorphic features, Myopia, Strabismus, High-arched palate, Low-set ears, Left UDT, Growth retardation, Speech delay, Flat feet						
28	М	2.5 Y	DD, Large ASD, TA, Short and bifid sternum, Hypotonia						
29	М	2 Y	DD, Large VSD, PS, Dextrocardia						
30	М	3 Y	DD, PDA, PS, PFO, Hypotonia, Bilateral ptosis, Strabismus, Bilateral UDT						
31	F	10 month	DD, TA, PDA, VSD, ASD, Dolicocephaly						
32	М	15.5 Y	ID, VSD, Epilepsy, Autism, Facial dysmorphic features, Retrognathia, Low-set ears, Joints hypermobility, Bilateral club feet, Polydactyly (right foot)						
33	F	1.5 Y	DD, ASD, Bilateral SNHL, Cleft palate, Facial dysmorphic features, Hip dislocation, Simple cyst (Left kidney)						
34	F	8 Y	ID, ASD, Autism, Hirschsprung, Bilateral inguinal hernia, Hypopigmentation of neck and back						
35	F	7 month	DD, ASD, Peripheral PS, Ascending aorta dilatation, Bilateral cataracts, Facial dysmorphic features, Left ptosis, Low set ears, Micrognathia						
36	М	2 Y	DD, PFO, Moderate bilateral SNHL, Hypotonia, Convulsion, Abnormal EEG, Hydrocephalus, Bilateral optic nerve atrophy						
37	F	1 Y	DD, Large VSD, PFO, Pulmonary hypertension, Macroglossia, Facial dysmorphic features, Flat feet						
38	F	12 Y	ID, TR, MVP, Macrocephaly, Ventriculomegaly (in MRI), Short stature, FTT, Ureteral stenosis, Facial dysmorphic features, Left ptosis, Photophobia, Ichthyosis, Sparse hair						
39	F	15 month	DD, Dilated right atrium and ventricle, Dilated pulmonary artery, Hypotonia, Chest deformity, Vermis hypoplasia, Prominent diameters frontal horns of lateral ventricles in brain sonography						
40	F	20 month	DD, ASD, PFO, Choanal atresia, Bilateral lower lid coloboma, Low-set ears, Brain hemiatrophy, Macroglossia						
41	F	10 Y	ID, Autism, Facial dysmorphic features, Microcephaly, Bilateral SNHL, Epilepsy, Polydactyly (4 limbs), Low birth weight, Growth retardation, Abnormal EEG, Hypotonia, Trigonocephaly, Short stature, Triangular face, Ptosis, Low-set ears, Posteriorly rotated ears, Retrognathia, Micrognathia, Downturned mouth, High-arched palate, Fecal/urinary incontinence, Feeding problems						
42	F	15 month	DD, VSD, ASD, RVH, Choanal atresia, Strabismus, Nasolacrimal duct obstruction, Bulbous nose						
43	F	21 Y	ID, Autism, ADHD, Insomnia, Severe obesity, Aggressiveness						
44	F	7 Y	ID, Autism, Bilateral lower lid coloboma, Facial dysmorphic features, Microcephaly, Retrognathia						
45	F	11 Y	ID, Autism, Diabetes mellitus						
46	F	10 Y	ID, Autism, Facial dysmorphic features, Low-set ears, Urinary incontinence, Right small kidney						
47	F	7.5 Y	ID, VSD, Epilepsy, Postaxial polydactyly (4 limbs), Syndactyly						
48	М	8.5 Y	ID, VSD, ASD, Facial dysmorphic features, Cleft lip and palate, Strabismus						
49	М	6.5 Y	ID, VSD, DD, Facial dysmorphic features, Left ureteral stenosis						
50	М	9 Y	ID, VSD, Epilepsy, Bilateral SNHL, Facial asymmetry, Strabismus, Microcephaly, Cleft lip/palate						

MCA; Multiple congenital anomalies, F; Female, M; Male, AR; Aortic regurgitation, AS; Aortic stenosis, ASD; Atrial septal defect, DD; Developmental delay, FTT; Failure to thrive, ID; Intellectual disability, IUGR; Intrauterine growth restriction, MVP; Mitral valve prolapse, PDA; Patent ductus arteriosus, PFO; Patent foramen ovale, PS; Pulmonic stenosis, RVH; Right ventricular hypertrophy, SNHL; Sensorineural hearing loss, SVAS; Supravalvular aortic stenosis, SVPS; Supravalvular pulmonic stenosis, TA; Tricuspid atresia, TGA; Transposition of the great arteries, TOF; Tetralogy of fallot, UDT; Undescended testis/ testes, VSD; Ventricular septal defect, ADHD; Attention deficit/hyperactivity disorder, EEG; Electroencephalography, TR; Tricuspid regurgitation, and MRI; Magnetic resonance imaging.

Chromosomal Abnormalities in Congenital Anomalies

Table 2: Frequency of main phenotypic manifestations of 50 patients
with MCA categorized by organ systems

Phenotype n (%)		Phenotype	n (%)			
Craniofacial	21 (62)*	Lower lid coloboma	3 (6)			
English duamorphic features	31 (62)	Optic nerve hypoplasia	2 (4)			
Miaraconholy	12 (26)	Glaucoma	1 (2)			
Macrocephaly	13 (20)	Cataract	1 (2)			
Claft lin/palata	3 (6)					
	3 (6)	Genitourinary system	19 (38)			
Doncocepnary	2 (4)	UDT	7 (14)			
Central nervous system	50 (100)	Urinary incontinence	3 (6)			
ID/DD	50 (100)	Small kidney	2 (4)			
Hypotonia	20 (40)	Urinary reflux	2 (4)			
Epilepsy	8 (16)	Small testes	1 (2)			
Corpus callosum agenesis	2 (4)	Hydrocele	1 (2)			
		Ureteral stenosis	1 (2)			
Cardiovascular system	45 (90)*	Rectovaginal fistula	1 (2)			
ASD	19 (38)	Simple kidney cyst	1 (2)			
VSD	15 (30)					
PS	8 (16)	Gastrointestinal system	5 (10)			
PDA	8 (16)	Dysphagia	2 (4)			
TOF	7 (14)	Fecal incontinence	2 (4)			
Pulmonary hypertension	4 (8)	Hirschsprung	1 (2)			
PFO	4 (8)		2 (()			
AS	2 (4)	Endocrine system	3 (6)			
		Hypothyroidism	2 (4)			
Musculoskeletal system	19 (38)*	Diabetes mellitus	1 (2)			
Short stature	4 (8)	Miscellaneous				
Polydactyly	4 (8)	Autism	8 (16)			
Club foot	3 (6)	ADHD	3 (6)			
Syndactyly	2 (4)	Growth retardation	3 (6)			
Brachydactyly	2 (4)	Choanal atresia	2 (4)			
Clinodactyly	2 (4)	Hypogammaglobulinemia	1 (2)			
Ear	19 (38)*	*; More than one abnormality may	be observed in one patient,			
SNHL	11 (22)	MCA; Multiple congenital anomalies, ID; Intellectual disability, DD; Developmental delay, ASD; Atrial septal defect, VSD; Ventricular septal				
Low-set ear (s)	9 (18)	defect, PS; Pulmonic stenosis, PDA; Patent ductus arteriosus, Tr Tetralogy of fallot, PFO; Patent foramen ovale, AS; Aortic stenosis, SN Sensorineural hearing loss, UDT; Undescended testis/testes, and ADI Attention deficit/hyperactivity disorder.				
Eye 16 (32)*						
Strabismus	6 (12)	The clinical findings for pati	ent 9 were compared with			
Hypertelorism	5 (10)	the previously reported patients in the literature ECARUCA database (http://www.ecaruca.ne				
Ptosis	4 (8)	Table 3.	()) III			
Visual defect	3 (6)	The cytogenetic and molecul	The cytogenetic and molecular cytogenetic results of 8			

Table 2: Continued

patients were reported in detail in Table 4.

Signs and symptoms	18p11.1- pter del (case ID 4621) (9)	18p11.21 p11.32 del (case ID 4772) (9)	18p11.21- pter del (10)	18p deletion (11)	18q21.3- q23 dup (12)	18q21.3- q23 dup (12)	Mosaic ring18 (13)	Mosaic ring18 (14)	Full trisomy 18 (15)	18p11.21p11.32 del and 18q21.31q23 dup (patient 9)
Growth retardation									+	+
ID/DD	+	+		+	+	+	+	+	+	+
Microcephaly	+								+	+
Defects of CNS									+	+
Speech disorder				+			+	+		+
Hypotonia	+		+		-		+	+	+	+
Heart defects						-	-		+	+
Optic nerve hypoplasia					-					+
Ptosis of eyelids	+	+		-			+		+	-
Strabismus			+	+	+		+			-
Hypertelorism			+	-	-		+		+	+
Down/up slanting- palpebral fissures		+	-		-	-	+		+	+
Low-set ears		+	+		-	-	+	+	+	+
Wide nasal bridge	+									+
Long/short philtrum					-		-	+	+	+
High-arched palate			-	+	-	+		+	+	+
Micrognathia			-		-	+	+		+	+
Clinodactyly			+		-		+	+	+	+
Rocker bottom feet									+	+
Short stature	+							+	+	+
IgA deficiency			-	+	-		+			+
IgG deficiency				-	-		+			+
IgM deficiency				-	-		+			+
Feeding problem	+									+
Genital malformations			-		-	-	-		+	+
Hernia		+			-	+	-			+

Table 3: The clinical features of patient 9 compared to those previously reported with 18p deletion, 18q duplication, mosaic ring(18), and full trisomy

ID; Intellectual disability, DD; Developmental delay, CNS; Central nervous system, +; Indicates presence, -; Indicates absence, and blank space; Indicates not available/not reported data.

Chromosomal Abnormalities in Congenital Anomalies

|--|

Patient	Cytogenetic band	Chromosomal sequence	Size	Del/Dup	Significant genes	Known syndromes	Inheritance
1	7q11.23	72,766,343-74,133,303	1.37 Mb	Del	22 OMIM genes ELN, LIMK1, RFC2, FKBP6, FZD9, STX1A, GTF2IRD1, BAZ1B	Williams-Beuren syndrome	De novo
5	5q35.2q35.3	175,559,373- 177,422,731	1.86 Mb	Del	25 OMIM genes including NSD1	Sotos syndrome	De novo
9	18p11.21p11.32	148,992-13,448,995	13.3 Mb	Del	44 OMIM genes TGIF1, LIPIN 2, LAMA1	-	De novo
	18q21.31q23	54,532,626-78,012,800	23.5 Mb	Dup	60 OMIM genes including MALT1, PIGN	-	
	18p11.21q21.31	(Mosaic ring, 37% of cells)	-	Dup	-	-	
13	22q11.21	18,706,023-21,561,492	2.86 Mb	Del	41 OMIM genes including TBX1	Digeorge syndrome	De novo
17	12q15-qter	-	-	Dup	HAND2	-	Maternal
	4q33	-	-	Del	-	-	
27	1q43q44	242,003,539-249,218,992	7.2 Mb	Del	19 OMIM genes including AKT3, NLRP3, HNRNPU, SMYD3 KIF26B, ZBTB18	Mental retardation- autosomal dominant 22 (MRD22)	De novo
	Xp22.33	60,814-601,612	541 Kb	Dup	4 OMIM genes including SHOX, PPP2R3B, PLCXD1, GTPBP6	-	
35	16p13.3	-	-	Del	CREBBP	Rubinstein- Taybi syndrome 1	De novo
41	3p26.3p25.3	93949_11504861	11.4 Mb	Del	SETD5, BRPF1, CHL1, CNTN4, SLC6A1, SLC6A11	3p deletion syndrome	De novo or Paternal
	10q26.3	135243049-135372492	130 Kb	Dup	CYP2E1, SYCE1	-	

MCA; Multiple congenital anomalies, Del; Deletion, and Dup; Duplication.

Patient 17, an 8.5-month-old boy born to nonconsanguineous parents with DD, infantile spasms and myoclonic jerks, abnormal EEG, PDA, ASD, Facial dysmorphic features, Hypotonia, and Corpus callosum hypoplasia (Table 1), showed an additional segment of 12q15-qter origin on 4q33 in karyotype analysis. The patient father's karyotype was normal, but his mother's karyotype showed a reciprocal balanced translocation between long arms of chromosomes 4 and 12 46,XX,t(4;12)(q33;q15). The clinical features of patient 17 were compared with those of the previously reported patients who had overlapping genotypes with patient 17. The information was obtained from the ECARUCA (9) and DECIPHER (16) databases (Table 5).

Signs and symptoms	12q21.31 - 12q21.32 dup patient ID 264283 (16)	12q21.32 - 12q23.1 dup patient ID 258582 (16)	12q23.3- q24.31 dup case ID 4588 (9)	12q24.1- q24.3 dup case ID 4053 (9)	4q33- qter del (17)	4q33- qter del (18)	4q33- qter del (18)	4q33- qter del (19)	12q15-qter dup and 4q33-qter del (patient 17)
Sex	М	F	М	F	М	F	М	М	М
Growth retardation			+		+			-	+
ID	+	+	+Severe		+	-	+	+	+
Seizures/Abnormal EEG		+					+	-	+
Microcephaly	+		+	+	+	+			+
Hypoplasia/agenesis of corpus callosum			+	+					+
Frontal bossing/high forehead									+
Hypotonia		+	+					-	+
Heart defects					-		+	+	+
Short neck			+	+	-			+	+
Hypertelorism			+		+			-	+
Epicanthal folds			+	+		+		-	+
Slanting palpebral fissures				Up		Up	Up	Up	Down
Malformed ears			+	+	-	+	+	+	+
Low-set ears			+	+			+	-	+
Micrognathia			+	+	+	+	+	-	-
Flat malar chin/flat mid- face							+	+	+
Thin upper/lower lip			+	+				+	-
High-arched palate		+						-	+
Down-turned corners of the mouth			+	+		+		+	-
Abnormality of the teeth		+	+					+	-
Abnormal palmar creases			+	+	-			+	+
Camptodactyly					-	+		-	+
Prominent/Bulbous nasal tip						+		-	+
Anteverted nares			+		+	+	+	+	+
Depressed/flat nasal bridge					+	+	-	-	+
High/prominent nasal bridge			+	+			+	-	-
Wide nasal bridge				+				+	+
Short stature			+	+	+	+		-	-
Foot deformity				+	+	+	+	+	+
Sacral dimple/sinus			+	+	+	+		-	+
Imperforated anus								-	+
Hearing loss								-	+Bilateral
Umbilical hernia									+
Cryptorchidism			+		+			-	+
Small kidneys									+

Table 5: The clinical features of patient 17 compared to the cases previously reported with partial 12q duplications and those with partial deletions of 4q

EEG; Electroencephalography, F; Female, ID; Intellectual disability, M; Male, +; Presence, -; Absence, and blank spaces indicate not available/not reported data.

Patient 41, a 10-year-old boy born to non-consanguineous healthy young parents with ID, autistic behavior, impaired social functioning, facial dysmorphic features, language delay, and microcephaly, bilateral SNHL, polydactyly (4 limbs), epilepsy, abnormal EEG, hypotonia, and trigonocephaly (Table 1), carried a terminal deletion of 3p in conventional cytogenetic characterization: 46, XY,del(3)(p25.3p26.3) (Fig.1C). The karyotype analysis of the proband's mother showed an apparently normal female karyotype, but the father's blood sample was unavailable. Further investigations using array-CGH confirmed 11.4 Mb terminal deletion of the short arm of chromosome 3 and about 130 Kb terminal duplication of the long arm of chromosome 10. Given the terminal 10g duplication, the probability that the 3p26.3p25.3 deletion was inherited from his father with 46,XX,t(3;10) (p26.3p25.3; q26.3) is high.

The karyotype of patient 27, an 18-year-old boy from non-consanguineous parents and born to a 31-year-old G7P6L6A1 mother at term, had been reported as a normal male in his medical documents. The result of further investigation using P245 Microdeletion Syndromes-1 kit was negative for the microdeletion/microduplication syndromes. The clinical features were ID, tetralogy of fallot (TOF), microcephaly, severe scoliosis, short stature, speech delay, and facial dysmorphic features (Table 1). In the conventional cytogenetic investigation repeated, a suspicion for terminal 1q deletion was observed. Array-CGH was performed and reported two inherited CNVs: A 7.2 Mb deletion of the terminal end of 1q43q44 and a 541 Kb duplication of Xp22.33. The array-CGH analyses performed for both of the parents were normal.

Patient 1 was a 14-month-old girl from healthy and young non-consanguineous parents born through normal vaginal delivery to a G1P1L1A0 mother at term. The clinical characteristics were ID, intrauterine growth restriction (IUGR), hypotonia, hypothyroidism, supravalvular aortic stenosis, supravalvular pulmonary stenosis, left visual defect, attention deficit/hyperactivity disorder, strabismus, long philtrum, and thick lips. Conventional cytogenetic analysis was normal. MLPA screening demonstrated a deletion of 7q11.23 consistent with Williams-Beuren syndrome. Further assays using array-CGH confirmed about 1.37 Mb deletion including 22 OMIM genes. Array-CGH analyses of the parents were normal.

Patient 5 was a 5.5-year-old girl, born through normal vaginal delivery to healthy and young non-consanguineous parents following a full-term pregnancy. Her mother was G2P1L1A1. She had suffered constipation for the first six months after birth. At the age of 14 months, the renal cortical scan revealed lesser activity in the lower pole of the left kidney because of pyelonephritis. During voiding cystourethrogram, severe urinary tract reflux was present in the left kidney. She also had a large ASD, ID, seizure attacks, autistic behavior, macrocephaly, dolicocephaly, frontal bossing, fish-shaped mouth, sparse hair, and joint laxity. She can say up to 5 words. The karyotype analysis

was normal. MLPA assay revealed a deletion of 5q35.3 consistent with Sotos syndrome. Further investigations using array-CGH confirmed 1.86 Mb deletion including 25 OMIM genes. Array-CGH analyses of the parents were normal.

Patient 13 was a 3-year-old boy who was the product of the first pregnancy and cesarean delivery of nonconsanguineous parents. The clinical features were widow's pick hair, down-slanting palpebral fissures, long eyelashes, deep set eyes, malformed, posteriorly rotated and low set ears, upward nose and nares, long philtrum, small mouth, high arched palate, high pitched cry, wheezing respiratory sounds, cardiac surgery scar, simian crease, 5th toe clinodactyly, umbilical hernia, sacral mongolian spot, overriding of 1st toe with 2nd toe, right lower limb paresis, joint contractures, right sided tippy-toed walking and bilateral undescended testes. Axial spiral CT-scan of the brain without contrast media showed bilateral pachygyria at frontotemporoparietal lobes due to abnormal neuronal migration. The karyotype analysis was normal. MLPA assay detected a deletion of 22q11.21 consistent with DiGeorge syndrome. Further investigations using array-CGH confirmed 2.86 Mb deletion including 41 OMIM genes, arr 22q11.21 (18,706,023-21,561,492) x1 dn. Array-CGH analyses of the parents were normal.

Patient 35 was a 7-month-old girl born through cesarean section to a healthy consanguineous (first cousin) couple. Her mother was G1P1L1A0. She had bilateral cataracts, hypertelorism, left ptosis, frontal bossing, low set ears, long philtrum, broad nasal bridge, micrognathia, a borderline lactic acid and normal creatinine in urine, ascending aorta dilatation, peripheral pulmonary stenosis, and small ASD. Conventional cytogenetic analysis showed a normal female karyotype. The MLPA technique indicated a deletion of 16p13.3 consistent with Rubinstein-Taybi syndrome. The sample assay was carried out twice. The parents' karyotype analyses and MLPA assays were normal.

Discussion

Based on several studies, the diagnostic yields of conventional cytogenetic studies in patients with MCA, global DD, and autism spectrum disorders, barring the known trisomy syndromes, have been reported to be about 3% (20). In this study, we identified chromosomal abnormalities, microdeletions, and microduplications for 8 patients (16%) using conventional karyotyping technique [3 patients (6%)], MLPA method [4 patients (8%)], and array-CGH [1 patient (2%)]. In addition to the sample size limitations, the adoption of inflexible criteria for selecting patients and the design type of the study could have contributed to the raising the diagnostic rate of this study.

Well-defined microdeletion syndromes were the most common cause of MCA in this study, consistent with the results of previously reported studies (21). The clinical features of four patients 1, 5, 13, and 35 are congruent with known microdeletion syndromes namely Williams-Beuren syndrome, Sotos syndrome, DiGeorge syndrome, and Rubinstein-Taybi syndrome, respectively. Consequently, they are not discussed in detail.

According to a hypothesis, two critical regions are within the long arm of chromosome 18 (18q), including 18q12.1-q21.2 and 18q22.3-qter. Duplication occurrence is essential in both proximal and distal regions in order to cause the typical clinical features of a trisomy 18 to be manifested (12). In patient 9, the former duplication is considered as a mosaic trisomy and the latter as a full trisomy.

As a chromosomal abnormality, partial deletion of 18p is more common than partial duplication of 18q (22). Mostly, a balanced translocation or inversion carried by one parent is the leading cause of partial trisomy of 18q (15). The real mechanism of mosaic ring 18 formation in patient 9 is unknown. Although his parents' karyotypes were normal, there was a recombinant pattern of pericentric inversion and an extra ring in the patient's karyotype. The possible mechanism for the formation of both derivative and ring chromosome 18 could, most probably, have initiated with an inverted chromosome 18 (p11.21q21.31), created during meiosis in the gamete of one parent (23). Postzygotic mitosis resulted in two partially q duplicated and partially p deleted recombinants (22). The genetic size of 18p is almost 16 Mb with a common breakpoint cluster located in the pericentromeric region with a length of 4 Mb. The partial deletion of 18p in this patient was inside the breakpoint cluster spanning 13.3 Mb. It should be noted that the 18q21.3 segment is one of the common fragile sites of chromosome 18 (24). One of the recombinants had seemed to have two breakpoints in 18q21.31 and 18p11.21 bands and the sticky ends of the segment between them fused and formed the ring chromosome 18. The broken segments were lost and some of the rings created were deleted in the next mitoses. The formation of ring chromosome 18 may be a novel escape mechanism in this patient with a pentasomy of 18q21.31qter segment (25).

Most clinical phenotypes of patient 9 overlapped with the clinical manifestations of partial 18p deletion, partial 18q duplication, and mosaic ring chromosome 18 syndromes. The phenotypic variations could be explained based on the locations and the lengths of the duplicated or deleted segments with a mosaic triplicated segment between them considering the co-occurrence of different types of chromosome 18 abnormalities in this patient.

Litzman et al. (13) reported an agammaglobulinemic 14-year-old girl with a mosaic ring chromosome 18. Her karyotype turned out to be: 45, XX,-18[5]/46, XX, dic r(18)[6]/46, XX, r(18)[89]. IgA deficiency/absence with normal ranges of IgG and IgM has been previously reported in association with 18p deletion (11). To the best of our knowledge, the proband is the first patient to be reported with partial monosomy of 18p, partial 18q duplication, and an extra mosaic ring chromosome 18 associated with hypogammaglobulinemia.

Based on the information obtained, no patient has been previously reported with partial duplication of 12q with the breakpoint at 12q15 to qter. However, although chromosome 4q deletions occur rarely, 4q33-qter deletions have been reported several times (17). It seems that some clinical features of patient 17 like hypoplasia of corpus callosum, hypotonia, and high-arched palate are also observed in the cases with partial 12q duplication. In contrast, some of the other physical characteristics such as heart defects, flat midface, camptodactyly, and depressed nasal bridge are consistent with phenotypic features of terminal deletion of 4q33-qter. However, a number of major anomalies in patient 17 including bilateral hearing loss, imperforated anus, umbilical hernia, and small kidneys have not been reported in the patients with partial 12q duplication or partial 4q deletion. The large size of 12q15-qter duplication could be the cause of these extra phenotypes. Homo sapiens HAND2 gene located on 4q34.1 has been known as the responsible gene whose proteins play a crucial role in developing ventricular chambers, cardiac morphogenesis, forming right ventricle and aortic arch arteries as well as developing limb and branchial arch. The haploinsufficiency of this gene in 4q deletion may justify inborn heart anomalies (i.e., VSD and ASD), growth retardation, ID, digital anomaly (i.e., clinodactyly and oligodactyly), and craniofacial dysmorphism including a cleft palate (26).

Approximately 11.4 Mb deletion of the distal segment of 3p containing 46 OMIM genes has led to severe and different phenotypes in patient 41.

The distal deletion of 3p is a rare contiguous gene syndrome with the variable spectrum of major anomalies such as developmental delay, growth retardation, autism, hearing loss, renal anomalies, heart defects, and craniofacial dysmorphic presentations. Given common facial dysmorphic features and ID among patients with intragenic sequence variants and microdeletions affecting *SETD5*, it has been suggested that this gene plays a vital role in ID and also has a significant contribution to the core phenotype of 3p deletion syndrome (27).

It seems that ID severity in patient 41 is due to the haploinsufficiency association of *SETD5* and *BRPF1* genes. *CHL1* gene deletion associated with mild ID and learning difficulties (28) may also contribute to this severity. Furthermore, some clinical manifestations specially ptosis and short stature may occur because of *BRPF1* gene deletion (29).

The autistic behavior of patient 41 may be because of *CNTN4* (contactin 4), previously introduced as a strong candidate gene associated with autism spectrum disorders (30). Two genes namely *SLC6A1* and *SLC6A11* encoding GABA transporter proteins have been proposed as possible candidates for seizures/EEG abnormalities, ataxia, and ID (31).

1q43q44 deletion in patient 27 with the size of 7.2 Mb included 126 HGNC and 19 OMIM genes. Of these

genes, *AKT3* gene haploinsufficiency has been linked with microcephaly in previously reported cases with 1q43q44 deletion syndrome (32). However, because of the presentation of microcephaly in the patients with intact *AKT3* gene, the other candidate genes such as *NLRP3*, *HNRNPU*, *SMYD3*, and *KIF26B* have been suggested to cause microcephaly (33). Nonetheless, *AKT3* deletion may explain the severity of microcephaly in patient 27.

Corpus callosum agenesis/hypoplasia and epilepsy are mostly associated with deletions of *ZBTB18* and *HNRNPU* genes in the patients with 1q43q44 deletion syndrome, respectively (34). However, none of these symptoms is demonstrated in the present case despite *ZBTB18* and *HNRNPU* disruptions. Recent studies have suggested that modifier genes or two-hit hypothesis may explain variable expressions among some patients with identical microdeletions. Another CNV is a disruptive singlebase-pair mutation in a related gene or an environmental event which influences the phenotype. These are all the examples of the second hit (35).

Patient 27 also had a duplication of about 541 Kb in Xp22.33 containing *PLCXD1*, *GTPBP6* and *PPP2R3B* genes as well as exons 1-3 and part of exon 4 of *SHOX* gene. *PPP2R3B* is one of the four major Ser/Thr phosphatases and negatively affects cell growth and division. *PPP2R3B* performs a regulatory control over the starting of DNA replication and its overexpression results in G1 phase cell cycle arrest (36).

There has been a variable impact on stature due to duplications of the region PAR1 containing the *SHOX* gene. Although some studies point to a correlation between *SHOX* gene deletions and short stature and Leri-Weill dyschondrosteosis, others have found some patients with the same clinical features who had partial or full duplications of *SHOX* gene. Bunyan et al. (37) reported a duplication of the exons 1-3 of the *SHOX* gene in a patient who had only short stature with no other clinical findings inherited from the proband's affected mother. Co-overexpression of *PPP2R3B* and *SHOX* genes seems to have a synergistic effect on our proband's short stature severity.

Conclusion

In this study, 50 patients with MCA were studied in terms of chromosomal abnormalities and microdeletion/ microduplication syndromes. Apatient was introduced with 46,XY, der(18)[12]/46,XY, der(18), +mar[8] dn as a novel case of MCA associated with hypogammaglobulinemia. Afterwards, given the highly complex and rare nature of the patient's chromosomal abnormality, a novel mechanism was proposed to explicate the formation of both derivative and ring chromosome 18. Furthermore, a new 12q abnormality was found which had not been reported previously. In addition, a novel association of an Xp22.33 duplication with 1q43q44 deletion syndrome was demonstrated. The phenotypic analysis of the introduced patients can be useful for further phenotype-genotype correlation studies.

Acknowledgements

We express our gratitude to the patients and their families for their participation and collaboration in this study. We would like to appreciate the assistance provided by the laboratory teams at the Genetics Research Center of University of Social Welfare and Rehabilitation Sciences and the Cytogenetics laboratory of Sarem Women's Hospital. We also extend our thanks to the deputy for research of the University of Social Welfare and Rehabilitation Sciences for funding this project. This work was supported by a grant from the deputy for research of the University of Social Welfare and Rehabilitation Sciences (grant number: 94/801/T/1/1871). The authors declare that they have no conflict of interest.

Authors' Contributions

F.B.; Supervision and grant application. F.B., H.N.; Study design. Y.S., S.A., E.A.-M., N.M., R.S.B., P.J., Z.C., R.M.G., A.K.; Patient recruitment and clinical evaluation. Y.S., S.A., A.K., P.J., F.H., Z.H.; Genetic counseling. A.M., S.B.; Experimental work and preparation of samples. A.M., F.B., S.G.F.; Karyotype analysis; A.M., F.B., S.G.F., A.N.; MLPA analysis. R.K., A.M., S.G.F.; Array-CGH analysis. A.M., F.B.; Writing first draft of manuscript. All the authors read and approved the final manuscript.

References

- Feldkamp ML, Carey JC, Byrne JLB, Krikov S, Botto LD. Etiology and clinical presentation of birth defects: population based study. BMJ. 2017; 357: j2249.
- World Health Organization. Congenital anomalies. 2017. Available from: http://www.who.int/mediacentre/factsheets/fs370/en/. (30 Nov 2017).
- Christianson A, Howson CP, Modell B. March of dimes. Global report on birth defect. The hidden toll of dying and disabled children. New York: March of Dimes Birth Defects Foundation; 2006.
- Agopian AJ, Evans JA, Lupo PJ. Analytic methods for evaluating patterns of multiple congenital anomalies in birth defect registries. Birth Defects Res. 2018; 110(1): 5-11.
- Kearney HM, Thorland EC, Brown KK, Quintero-Rivera F, South ST; Working Group of the American College of Medical Genetics Laboratory Quality Assurance Committee. American College of Medical Genetics standards and guidelines for interpretation and reporting of postnatal constitutional copy number variants. Genet Med. 2011; 13(7): 680-685.
- Barch MJ, Knutsen T, Spurbeck JL; Association of genetic technologists. The AGT cytogenetics laboratory manual. 3rd ed. Philadelphia: Lippincott-Raven Publishers; 1997.
- McGowan-Jordan J, Simons A, Schmid M. ISCN 2016-An International system for human cytogenetic nomenclature. Basel: Karger; 2016.
- 8. Liehr T. Fluorescence in situ hybridization (FISH)-application guide. 2nd ed. Berlin, Heidelberg: Springer Berlin Heidelberg; 2017.
- Vulto-van Silfhout AT, van Ravenswaaij CM, Hehir-Kwa JY, Verwiel ET, Dirks R, van Vooren S, et al. An update on ECARUCA, the European cytogeneticists association register of unbalanced chromosome aberrations. Eur J Med Genet. 2013; 56(9): 471-474.
- Wester U, Bondeson ML, Edeby C, Annerén G. Clinical and molecular characterization of individuals with 18p deletion: a genotype-phenotype correlation. Am J Med Genet A. 2006; 140(11): 1164-1171.
- 11. Fischer P, Golob E, Friedrich F, Kunze-Mühl E, Doleschel W, Aichmair H. Autosomal deletion syndrome 46,XX,18p-: a new case re-

port with absence of IgA in serum. J Med Genet. 1970; 7(1): 91-98.

- Boghosian-Sell L, Mewar R, Harrison W, Shapiro RM, Zackai EH, Carey J, et al. Molecular mapping of the Edwards syndrome phenotype to two noncontiguous regions on chromosome 18. Am J Hum Genet. 1994; 55(3): 476-483.
- Litzman J, Bryšová V, Gaillyová R, Thon V, Pijáčková A, Michalová K, et al. Agammaglobulinaemia in a girl with a mosaic of ring 18 chromosome. J Paediatr Child Health. 1998; 34(1): 92-94.
- Bagherizadeh E, Behjati F, Saberi S, Shafeghati Y. Prenatal diagnosis in a mentally retarded woman with mosaic ring chromosome 18. Indian J Hum Genet. 2011; 17(2): 111-113.
- 15. Cereda A, Carey JC. The trisomy 18 syndrome. Orphanet J Rare Dis. 2012; 7: 81.
- Firth HV, Richards SM, Bevan AP, Clayton S, Corpas M, Rajan D, et al. DECIPHER: Database of chromosomal imbalance and phenotype in humans using ensembl resources. Am J Hum Genet. 2009; 84(4): 524-533.
- 17. Stamberg J, Jabs EW, Elias E. Terminal deletion(4)(q33) in a male infant. Clin Genet. 1982; 21(2): 125-129.
- Tomkins DJ, Hunter AG, Uchida IA, Roberts MH. Two children with deletion of the long arm of chromosome 4 with breakpoint at band q33. Clin Genet. 1982; 22(6): 348-355.
- Borochowitz Z, Shalev SA, Yehudai I, Bar-El H, Dar H, Tirosh E. Deletion (4)(q33-->qter): a case report and review of the literature. J Child Neurol. 1997; 12(5): 335-337.
- Hochstenbach R, Buizer-Voskamp JE, Vorstman JA, Ophoff RA. Genome arrays for the detection of copy number variations in idiopathic mental retardation, idiopathic generalized epilepsy and neuropsychiatric disorders: lessons for diagnostic workflow and research. Cytogenet Genome Res. 2011; 135(3-4): 174-202.
- Rauch A, Hoyer J, Guth S, Zweier C, Kraus C, Becker C, et al. Diagnostic yield of various genetic approaches in patients with unexplained developmental delay or mental retardation. Am J Med Genet A. 2006; 140(19): 2063-2074.
- Zamani AG, Acar A, Durakbasi-Dursun G, Yildirim MS, Ceylaner S, Tuncez E. Recurrent proximal 18p monosomy and 18q trisomy in a family due to a pericentric inversion. Am J Med Genet Part A. 2014; 164A(5): 1239-1244.
- Gereltzul E, Baba Y, Suda N, Shiga M, Inoue MS, Tsuji M, et al. Case report of de novo dup(18p)/del(18q) and r(18) mosaicism. J Hum Genet. 2008; 53(10): 941-946.
- 24. Debacker K, Kooy RF. Fragile sites and human disease. Hum Mol Genet. 2007; 16 Spec No. 2: R150-R158.
- 25. Knijnenburg J, van Haeringen A, Hansson KB, Lankester A, Smit MJ, Belfroid RD, et al. Ring chromosome formation as a novel escape mechanism in patients with inverted duplication and terminal

deletion. Eur J Hum Genet. 2007; 15(5): 548-555.

- Tamura M, Amano T, Shiroishi T. The Hand2 gene dosage effect in developmental defects and human congenital disorders. Curr Top Dev Biol. 2014; 110: 129-152.
- Kuechler A, Zink AM, Wieland T, Lüdecke HJ, Cremer K, Salviati L, et al. Loss-of-function variants of SETD5 cause intellectual disability and the core phenotype of microdeletion 3p25.3 syndrome. Eur J Hum Genet. 2015; 23(6): 753-760.
- 28. Cuoco C, Ronchetto P, Gimelli S, Béna F, Divizia MT, Lerone M, et al. Microarray based analysis of an inherited terminal 3p26.3 deletion, containing only the CHL1 gene, from a normal father to his two affected children. Orphanet J Rare Dis. 2011; 6: 12.
- Mattioli F, Schaefer E, Magee A, Mark P, Mancini GM, Dieterich K, et al. Mutations in histone acetylase modifier BRPF1 cause an autosomal-dominant form of intellectual disability with associated ptosis. Am J Hum Genet. 2017; 100(1): 105-116.
- Bakkaloglu B, O'Roak BJ, Louvi A, Gupta AR, Abelson JF, Morgan TM, et al. Molecular cytogenetic analysis and resequencing of contactin associated protein-like 2 in autism spectrum disorders. Am J Hum Genet. 2008; 82(1): 165-173.
- Dikow N, Maas B, Karch S, Granzow M, Janssen JWG, Jauch A, et al. 3p25.3 microdeletion of GABA transporters SLC6A1 and SLC6A11 results in intellectual disability, epilepsy and stereotypic behavior. Am J Med Genet Part A. 2014; 164A(12): 3061-3068.
- Ballif BC, Rosenfeld JA, Traylor R, Theisen A, Bader PI, Ladda RL, et al. High-resolution array CGH defines critical regions and candidate genes for microcephaly, abnormalities of the corpus callosum, and seizure phenotypes in patients with microdeletions of 1q43q44. Hum Genet. 2012; 131(1): 145-156.
- Raun N, Mailo J, Spinelli E, He X, McAvena S, Brand L, et al. Quantitative phenotypic and network analysis of 1q44 microdeletion for microcephaly. Am J Med Genet A. 2017; 173(4): 972-977.
- Depienne C, Nava Ć, Keren B, Heide S, Rastetter A, Passemard S, et al. Genetic and phenotypic dissection of 1q43q44 microdeletion syndrome and neurodevelopmental phenotypes associated with mutations in ZBTB18 and HNRNPU. Hum Genet. 2017; 136(4): 463-479.
- 35. Veltman JA, Brunner HG. Understanding variable expressivity in microdeletion syndromes. Nat Genet. 2010; 42(3): 192-193.
- Yan Z, Fedorov SA, Mumby MC, Williams RS. PR48, a novel regulatory subunit of protein phosphatase 2A interacts with Cdc6 and modulates DNA replication in human cells. Mol Cell Biol. 2000; 20(3): 1021-1029.
- Bunyan DJ, Baker KR, Harvey JF, Thomas NS. Diagnostic screening identifies a wide range of mutations involving the SHOX gene, including a common 47.5 kb deletion 160 kb downstream with a variable phenotypic effect. Am J Med Genet A. 2013; 161A(6): 1329-1338.

Mitochondrial Polymorphisms, in The D-Loop Area, Are Associated with Brain Tumors

Donya Altafi, M.Sc.^{1*}, Soha Sadeghi, M.Sc.¹, Hamed Hojatian, M.Sc.¹, Maryam Torabi Afra, M.Sc.¹, Safoura Pakizeh Kar, M.Sc.², Mojtaba Gorji, Ph.D.³, Massoud Houshmand, Ph.D.^{4, 5*}

Molecular Biology Department, NourDanesh Institute of Higher Education, Esfahan, Iran
 Department of Biology, International University of Guilan, Guilan, Iran
 Department of Hematology and Oncology, Lorestan Medical University, Lorestan, Iran
 Department of Medical Genetics, National Institutes for Genetic Engineering and Biotechnology, Tehran, Iran
 Research Center, Knowledge University, Erbil, Kurdistan Region, Iraq

*Corresponding Addresses: P.O.Box: 1483614681, Molecular Biology Department, NourDanesh Institute of Higher Education, Esfahan, Iran

P.O.Box: 14965/161, Department of Medical Genetics, National Institutes for Genetic Engineering and Biotechnology, Tehran, Iran Emails: donya.altafi@gmail.com, massoudh@nigeb.ac.ir

Received: 1/April/2018, Accepted: 29/October/2018

Abstract — Objective: This study was carried out to evaluate the relationship between mtDNA D-loop variations and the pathogenesis of a brain tumor.

Materials and Methods: In this experimental study, 25 specimens of brain tumor tissue with their adjacent tissues from patients and 454 blood samples from different ethnic groups of the Iranian population, as the control group, were analysed by the polymerase chain reaction (PCR)-sequencing method.

Results: Thirty-six variations of the D-loop area were observed in brain tumor tissues as well as the adjacent normal tissues. A significant difference of A750G (P=0.046), T15936C (P=0.013), C15884G (P=0.013), C16069T (P=0.049), T16126C (P=0.006), C16186T (P=0.022), T16189C (P=0.041), C16193T (P=0.045), C16223T (P=0.001), T16224C (P=0.013), C16234T (P=0.013), G16274A (P=0.009), T16311C (P=0.038), C16327T (P=0.045), C16355T (P=0.003), T16362C (P=0.006), G16384A (P=0.042), G16392A (P=0.013), G16394A (P=0.013), and G16477A (P=0.013) variants was found between the patients and the controls.

Conclusion: The results indicated individuals with C16069T [odds ratio (OR): 2.048], T16126C (OR: 2.226), C16186T (OR: 3.586), G16274A (OR: 4.831), C16355T (OR: 7.322), and T16362C (OR: 6.682) variants with an OR more than one are probably associated with a brain tumor. However, given the multifactorial nature of cancer, more investigation needs to be done to confirm this association.

Keywords: Brain Tumor, D-Loop, Mitochondrial DNA

Cell Journal(Yakhteh), Vol 21, No 3, October-December (Autumn) 2019, Pages: 350-356

Citation: Altafi D, Sadeghi S, Hojatian H, Torabi Afra M, Pakizeh Kar S, Gorji M, Houshmand M. Mitochondrial polymorphisms, in the D-loop area, are associated with brain tumors. Cell J. 2019; 21(3): 350-356. doi: 10.22074/cellj.2019.5947.

Introduction

Brain tumors refer to all tumors in the central spinal canal or inside the cranium. All brain tumors are innately serious and fatal because of their infiltrative and invasive features in the confines of the intracranial cavity. According to the American Cancer Society, death estimation of Brain and other nervous system tumors is 6,150 (2%) per 100,000 in 2013. In 2013, 1310 cancer deaths amongst children between 0 to 14 years old were reported. Approximately, 25% of all cancers in children are due to brain and other central nervous system tumors (1-3).

The human mitochondrial DNA (mtDNA) is a 16,569 base-pair-long double-strand DNA. Mitochondrial genome codes thirteen pivotal polypeptides of the respiratory chain enzyme complexes, which are located in the inner membrane of the mitochondria, along with two ribosomal RNAs (rRNAs) and twenty-two transfer RNAs (tRNAs) that are crucial for protein synthesis and intramitochondrial translation, respectively (4). There are 10^3 to 10^4 copies of mtDNA per human cell (5). A 1.1 kb of noncoding displacement loop (D-loop) region is situated between the aforementioned genes. This region consists of heavy-strand and light-strand promoter regions which are essential for the mitochondrial transcription and replication process (4, 6, 7).

Due to insufficient DNA repair mechanisms, the absence of protective histone proteins, and a considerable level of the reactive oxygen species (ROS) production during oxidative phosphorylation (OXPHOS), mitochondrial DNA is susceptible to oxidative damage and carries significantly more mutations than nuclear DNA. In mtDNA, oxidative damages and the subsequent mutations can accumulate at a rate of 10-fold faster than nuclear DNA (8).

In the past, a myriad of somatic mtDNA mutations, including insertions, deletions, point mutations, and altered mtDNA copy numbers, have been detected in numerous human cancers (9). In the mitochondrial genome, some of these mutations lead to functional alterations in its encoded proteins or missense mRNA transcription (10). In the coding region, the common deletion region (CD) (4,977 bp deletion) is the most frequent (11). It has been shown that large deletions in the mitochondrial genome are accumulated in subjects with heteroplasmic mtDNA mutations and healthy elders (12).

The somatic variations in the D-loop area may be associated with the diminution of mtDNA copy numbers as well (13). The D-loop area of Mitochondrial DNA is a polymorphic region (14). The most of the mtDNA mutations occurring in D-loop are linked to human malignancies (13). Therefore, studying mtDNA mutations in various tumor cells is pivotal for understanding the relationship between mtDNA D-loop mutations and the initiation and progression of a tumor (15).

Materials and Methods

Subjects

In this experimental study, 25 samples of brain tumors and adjacent tissues (2 women and 23 men in the age range of 28 to 70 years old) were collected (Table 1). Furthermore, 454 blood samples from healthy controls were included; these samples are from 100 random individuals, belonging to17 ethnicities of the Iranian population. All of the samples were collected from the Tehran Special Medical Center. Tumor tissues and adjacent ones were quickly frozen by liquid nitrogen and transferred to a -80°C freezer. The cancer diagnosis was confirmed via histological analysis. In the control group, the exclusion criterion was metabolic diseases, history of cancer, and any mitochondrial DNA related diseases which might affect the mtDNA.

Sample	Age	Clinical data	Diagnosis	Gender
1	53	Left frontal brain tumor	Astrocytoma	Male
2	53	Right fronto-temporal brain tumor	Giant Cell Glioblastoma	Male
3	40	A right parieto-occipital brain tumor	Oligodendroglioma	Male
4	48	Right temporal brain tumor	Astrocytoma	Male
5	58	Left frontotemporal brain tumor	Astrocytoma	Male
6	33	Intraventicular brain tumor	Ependymoma	Male
7	56	Right temporal brain tumor	Astrocytoma	Male
8	51	Right temporal brain tumor	Glioblastoma multiform	Male
9	55	Left temporal brain tumor	Astrocytoma	Male
10	57	Right frontoparietal brain tumor	Glioblastoma multiform	Male
11	65	Left temporal brain tumor	Astrocytoma	Male
12	42	Right parieto-occipital brain tumor	Oligodendroglioma	Male
13	70	Right frontoparietal brain tumor	Glioblastoma multiform	Female
14	54	Right frontal brain tumor	Glioblastoma multiform	Male
15	58	A cerebral tumor	Glioblastoma multiform	Male
16	53	Right temporal brain tumor	Glioblastoma multiform	Male
17	63	Right temporal brain tumor	Glioblastoma multiform	Male
18	40	A cerebral tumor	Glioblastoma multiform	Male
19	28	Left frontal brain tumor	Glioblastoma multiform	Male
20	55	A right frontoparietal brain tumor	Oligodendroglioma	Male
21	63	Right frontal brain tumor	Glioblastoma multiform	Male
22	32	Left frontal cerebral tumor	Glioblastoma multiform	Male
23	32	Cerebral tumor	Astrocytoma	Male
24	56	Right temporal brain tumor	Astrocytoma	Female
25	36	Left temporal brain tumor	Astrocytoma	Male

Table 1: Age and histological features of brain tumors in patients

DNA extraction

Genomic DNA of the tumor and the adjacent tissues were isolated by DNA extraction kit (Qiagen, Netherlands) and blood samples of the controls. which were collected in Ethylenediaminetetraacetic acid (EDTA)-containing tubes, were extracted using Diatom DNA extraction kit (Gene Fanavaran, Iran) and their quantity and quality were analyzed by NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific) and agarose gel electrophoresis, respectively.

Mitochondrial D-loop genotyping

Primers for amplifying of the mtDNA D-loop region were adapted from Seyedhassani et al. (16) and Shakhssalim et al. (17).

The volume of a PCR reaction was 25 μ l and contained 50-100 ng of the DNA, 0.8 μ l of the primers, 0.8 μ l of MgCl₂, 0.5 μ l of dNTPs 10 mM, 2.5 μ l of PCR buffer (10X), and 0.3 μ l of Taq DNA polymerase (Roche Applied Sciences, Germany). Moreover, the PCR procedure was carried out according to the following protocol: pre-denaturation phase (5 minutes at 94°C), followed by 35 cycles of the shorter denaturation phase (50 seconds at 94°C), annealing step (50 seconds at 55°C), the extension step (50 seconds at 72°C), The final stage is the extension phase for 10 minutes at 72°C. Each amplified fragment subsequently was sequenced using an ABI PRISM 3730 sequence analyzer (Macrogen, Korea). The acquired sequencing data were evaluated using Finch TV.

Statistical analysis

Finch TV version 1.4.0 was used to edit and align the sequences. The NCBI blast was used as a reference. The statistical analysis was carried out using IBM SPSS statistics for windows, version 24.0 (IBM Corp., Armonk, NY, USA). The chi-square was used to evaluate the deviation from the Hardy-Weinberg equilibrium. This association between a brain tumor and D-loop variants was evaluated using SPSS version 22. A P<0.05 was considered statistically significant.

Results

In the present study, tissue samples from 25 subjects with brain tumors and 454 blood samples from the control group were genotyped for D-loop region variants. The subsequent results showed 36 variations in the D-loop area (Table 2), most of which were already reported under MITOMAP. One hundred and fifteen variations of the D-loop region were found in the control groups (Table 3). However, 34 mutations in the patients and 103 mutations in the control were reported in advance, and 2 mutations in the patients and 12 mutations in the control were novel mutations (Table 4). All variations were single nucleotide substitutions and the majority of them were C \rightarrow T. (33.33%) T \rightarrow C (30.55%) G \rightarrow A (22.22%) $A \rightarrow C$ (2.77%) $C \rightarrow G$ (2.77%). The D-loop region variant distributions in the controls and the patients are shown in Table S1 (See Supplementary Online Information at www. celljournal.org). The significant associations between variants and brain tumor risk (P<0.05) are shown in Table 5.

 Table 2: List of D-loop region variants in the patient group (tissue sample)

A152G	T195C	T204C	G207A	A263G	T489C	A750G	C15884G	G15928A	T15936C*
C16069T	T16126C	G16145A	C16148T	T16172C	C16186T	T16189C	C16193T	C16223T	T16224C
C16234T	C16256T	C16261T	C16270T	G16274A	C16292T	T16311C	A16318C	C16327T	C16355T
T16362C	G16384A	G16392A	G16394A	G16477A	T16519C				

*; Shows unreported mutations near the D-loop region.

Table 3: List of D-loop region variants in control	l group (blood sample)
--	------------------------

A152G	T195C	T204C	G207A	A263G	T489C	A750G	A15908G	T15924C	C15927T
C15928T	G15946A	G16004C*	A16017G	A16069G	G16071A	A16075G	C16084T	A16086G	A16087G*
A16093G	G16095A	G16114A	G16124A*	A16126G	A16128G*	C16129T	G16131A*	C16145T	G16148A
C16149T	G16150A	T16162C	T16163C	A16172G	G16174A	G16176A	A16177G	A16179G	G16184A
G16186A	C16187T	A16189G	G16192A	G16193A	G16197A	G16201A	T16203C	A16209G	A16217G
A16223G	G16233A	G16236A	G16242A	A16243G	G16245A	A16248G	A16249G	G16256A	G16257A
G16259A	G16261A	G16262A	T16265C	C16266T	G16270A	A16271G	A16272G	C16274T	G16278A
A16286G	G16287A	A16289G	G16290A	G16291A	G16292A	T16293G	G16294A	G16295A	G16296A
A16299G	A16304G	T16309C	A16311G	G16315A*	T16316C	T16318A	C16319T	A16323G	A16325G
A16334G	C16339T	A16342G	T16343C	A16344G*	A16352G	G16354T	G16355A	A16356G	A16359G
G16360A	A16362G	G16364A	C16390T	C16391T	C16398T	T16399C	A16468G	G16478C	G16481A*
G16488A	T16497C	T16519C	C16526T	G16527A					

*; Shows unreported variations in D-loop region and adjacent sequences.

Table 4: List of unreported variants in patient and control groups

					anto in patient		<u>«þ</u> .		
A15499G	G16004C	A16087G	G16124A	A16128G	G16131A	A16179G	G16233A	A16286G	G16315A
A16344G	G16481A								

Variant	Patients (%)	Control (%)	P value	Chi-square tests	Odds ratio
T15936C	6.25	0	0.013	6.186 ^a	0.94
C15884G	6.25	0	0.013	6.186 ^a	0.94
G15928A	6.25	6.38	1	0.000ª	1
C16069T	25	13.87	0.049	3.854ª	2.048
T16126C	50	30.83	0.006	7.490ª	2.226
G16145A	18.75	12.99	0.247	1.339ª	1.57
C16148T	6.25	3.96	0.516	0.421ª	1.532
T16172C	6.25	4.18	0.516	0.421ª	1.532
C16186T	12.5	3.52	0.022	5.207ª	3.586
T16189C	12.5	22.9	0.041	4.190 ^a	0.457
C16193T	6.25	1.32	0.045	3.701 ^a	6.319
C16223T	12.5	31.71	0.001	10.351ª	0.318
T16224C	6.25	0.22	0.013	6.186 ^a	0.94
C16234T	6.25	0.88	0.013	6.186 ^a	0.94
C16256T	6.25	4.4	0.516	0.421ª	1.532
C16261T	18.75	10.57	0.113	2.510 ^a	1.898
C16270T	6.25	2.6	0.306	1.047 ^a	2.064
G16274A	12.5	2.86	0.009	6.793 ^a	4.831
C16292T	6.25	2.64	0.306	1.047ª	2.064
T16311C	6.25	14.75	0.038	4.310 ^a	0.362
A16318C	6.25	4.62	0.756	0.96 ^a	1.213
C16327T	6.25	0.66	0.045	3.701 ^a	6.319
C16355T	12.5	2.2	0.003	8.721ª	7.322
T16362C	18.75	9.25	0.006	7.680ª	6.682
G16384A	6.25	0	0.042	4.153 ^a	2.372
G16392A	6.25	0.22	0.013	6.186 ^a	0.94
G16394A	6.25	0	0.013	6.186 ^a	0.94
G16477A	6.25	0	0.013	6.186 ^a	0.94
T16519C	56.25	62.55	0.313	1.017ª	0.747
A152G	32	30.8	0.87	0.023ª	1.047
T195C	12	21.4	0.08	2.94ª	0.5
T204C	12	9.3	0.48	0.47^{a}	1.37
G207A	12	7	0.22	1.45ª	1.81
A263G	100	100	0	0	0
T489C	24	19.1	0.38	0.741ª	1.34
A750G	84	93.3	0.046	3.979ª	0.39

^a; 0 blocks (0.0%) have considered rate less than 5. The minimum envisaged rate is 31.50.

Discussion

Studies have shown that the accumulation of mitochondrial DNA variations and mitochondrial instability contributes to several diseases, including cancer. The mitochondrial D-loop area has pivotal functions in transcription, replication, and mtDNA organization. Presence of two critical factors makes mitochondrial genome prone to different mutations: ROS and low-level of DNA repair system in mitochondria (18). However, the D-loop region is even more susceptible to the mutations than the rest of the human mitochondrial genome (12).

It has been demonstrated that the expansion of poly-C repeats in highly variable regions (HVR), adjacent to the D-loop and tRNA phenylalanine, increases the copy number; homoplasmy and loss of mitochondrial heteroplasmy act like cancer suppressor genes and will cause brain cancer and other cancers in humans (19).

Recent studies demonstrate that mitochondrial D-loop mutations play an important role in Huntington's disease (20), as well as various cancers, including brain tumors. Consequently, in this survey was hypothesised that particular mutations in the D-loop region might be related with brain tumor risk.

Twenty-five tissue samples of brain tumors were assessed. As it is not possible to use brain tissues of healthy individuals, we used 454 blood samples as the control. A significant difference between the cancer tissue and the healthy tissue was observed. Also, we studied the mutations reported in MITOMAP in more detail.

It has been proven that D-loop variants are associated with different disease: T195C variant with glaucoma (21) and G15884C variant with pancreatic cancer (22) and G15928A with MS, recurrent idiopathic abortion and AD (23) and C16069T with bladder cancer (24) and T16126C with Huntington's disease (20) and C16172T with head and neck cancer (25) and T16189C with prostate cancer (26) and C16193T with ovarian cancer (27) and C16223T with cancer, and Huntington's (20) and G16274A linked to prostate cancer (28) and C16292T related with breast and ovarian cancer (27) and T16311C with prostate cancer (28) and T16519C with glioblastoma, migraines (29). Although T204C (30), G207A (28), A263G (31), T489C (25), A750G (21), G16145A (16), C16148T (32), C16186T (33), T16224C, C16234T, C16256T (34), C16270T, C16261T (35, 36), C16327T (37), C16355T (38), T16362C (39), G16384A (16), G16392A, G16394A, G16477A (40), were associated with various cancers and were reported in MITOMAP but they were not associated with the disease.

In this experimental study, A750G, T15936C, C15884G, C16069T, T16126C, C16186T, T16189C, C16193T, C16223T, T16224C, C16234T, G16274A, T16311C, C16327T, C16355T, T16362C, G16384A,

G16392A, G16394A, G16477A variants had statistically significant different frequencies between patient and control groups. Thus, it seems that they are associated with brain tumors. Among the mentioned polymorphisms, C16069T, T16126C, C16186T, G16274A, C16355T, and T16362C had a greater odd ratio (OR) than the rest. C16355T polymorphism had the most significant association with the disease in risk.

It should be noted that A152G, T204C, G207A, A263G, T489C, A750G, G15928A, G16145A, C16148T, T16172C, C16256T, C16261T, C16270T, C16292T, A16318C, and T16519C polymorphisms were not associated with the disease risk. Although, they have significant roles in other disease pathogenesis as they have been reported on MITOMAP.

In Lorr ethnicity, T15936C, C15884G, G15928A, T16126C, C16148T, T16172C, C16186T, C16186T, C16193T, C16223T, T16224C, C16234T, C16256T, C16292T, T16311C, T16362C, G16384A, G16392A, G16394A, and G16477A polymorphisms were associated with brain tumors. T16126C and T16362C polymorphisms had a higher odds ratio than the rest which indicates they are more associated with this disease.

C16069T, G16145A, T16189C, C16261T, C16270T, A16318C, C16327T, and T16519C polymorphisms were not associated with the disease in Lorr ethnicity. However, the mentioned polymorphisms are associated with other disease pathogenesis as they reported on MITOMAP.

In Fars ethnicity, T15936C, C15884G, C16069T, T16126C, C16193T, T16224C, C16234T, C16261T, C16292T, C16327T, C16355T, G16384A, G16392A,G16394A, and G16477A variants were associated with the disease, C16069T, T16126C, C16186T, G16274A, C16355T, and T16362C polymorphisms had greater odds ratio than the rest, and G15928A, G16145A, C16148T, T16172C, C16186T, T16189C, C16223T, C16256T, C16270T, G16274A, T16311C, A16318C, T16362C, and T16519C variants were not associated with the disease.

Conclusion

Base on the odds ratio result, C16069T, T16126C, C16186T, G16274A, C16355T, and T16362C alterations were significantly associated with brain tumor. Among these variants, C16355T with odds ratio 7.322 was the strongest one.

Acknowledgements

This investigation was supported by the National Institute of Genetic Engineering and Biotechnology, Tehran, Iran and NourDanesh Institute of Higher Education, Esfahan, Iran. There is not any fund to carry out this project. All authors have neither pecuniary nor ethical conflict of interests.

Authors' Contributions

D.A.; Contributed to sample collection, statistical analysis, sample processing, writing the manuscript and providing laboratory materials. S.S.; Contributed to all experimental work, data and statistical analysis, interpretation of data, and editing the final manuscript. H.H.; Contributed to statistical analysis and all experimental work. M.T.A., S.P.K., M.G.; Contributed to sample collection and all experimental work. M.H.; Contributed to providing laboratory equipment and materials and was responsible for overall supervision. All authors read and approved the final manuscript.

References

- Helfer JL, Wen PY, Blakeley J, Gilbert MR, Armstrong TS. Report of the Jumpstarting Brain Tumor Drug Development Coalition and FDA clinical trials clinical outcome assessment endpoints workshop (October 15, 2014, Bethesda MD). Neuro Oncol. 2016; 18 Suppl 2: ii26-ii36.
- Ostrom QT, Gittleman H, de Blank PM, Finlay JL, Gurney JG, McKean-Cowdin R, et al. American brain tumor association adolescent and young adult primary brain and central nervous system tumors diagnosed in the United States in 2008-2012. Neuro Oncol. 2016; 18 Suppl 1: i1-i50.
- Ostrom QT, Gittleman H, Liao P, Vecchione-Koval T, Wolinsky Y, Kruchko C, et al. CBTRUS statistical report: primary brain and other central nervous system tumors diagnosed in the United States in 2010-2014. Neuro Oncol. 2017; 19(suppl_5): v1-v88.
- Anderson S, Bankier AT, Barrell BG, de Bruijn MH, Coulson AR, Drouin J, et al. Sequence and organization of the human mitochondrial genome. Nature. 1981; 290(5806): 457-465.
- Bogenhagen D, Clayton DA. The number of mitochondrial deoxyribonucleic acid genomes in mouse L and human HeLa cells. Quantitative isolation of mitochondrial deoxyribonucleic acid. J Biol Chem. 1974; 249(24): 7991-7995.
- Walberg MW, Clayton DA. Sequence and properties of the human KB cell and mouse L cell D-loop regions of mitochondrial DNA. Nucleic Acids Res. 1981; 9(20): 5411-5421.
- Clayton DA. Replication and transcription of vertebrate mitochondrial DNA. Annu Rev Cell Biol. 1991; 7: 453-478.
- Croteau DL, Bohr VA. Repair of oxidative damage to nuclear and mitochondrial DNA in mammalian cells. J Biol Chem. 1997; 272(41): 25409-25412.
- Yin PH, Lee HC, Chau GY, Wu YT, Li SH, Lui WY, et al. Alterations of the copy number and deletion of mitochondrial DNA in human hepatocellular carcinoma. Br J Cancer. 2004; 90(12): 2390-2396.
- Meierhofer D, Mayr JA, Fink K, Schmeller N, Kofler B, Sperl W. Mitochondrial DNA mutations in renal cell carcinomas revealed no general impact on energy metabolism. Br J Cancer. 2006; 94(2): 268-274.
- Shoffner JM, Lott MT, Voljavec AS, Soueidan SA, Costigan DA, Wallace DC. Spontaneous Kearns-Sayre/chronic external ophthalmoplegia plus syndrome associated with a mitochondrial DNA deletion: a slip-replication model and metabolic therapy. Proc Natl Acad Sci USA. 1989; 86(20): 7952-7956.
- Wallace DC. Mitochondrial DNA in aging and disease. Sci Am. 1997; 277(2): 40-47.
- Bianchi MS, Bianchi NO, Bailliet G. Mitochondrial DNA mutations in normal and tumor tissues from breast cancer patients. Cytogenet Cell Genet. 1995; 71(1): 99-103.
- Corral-Debrinski M, Horton T, Lott MT, Shoffner JM, Beal MF, Wallace DC. Mitochondrial DNA deletions in human brain: regional variability and increase with advanced age. Nat Genet. 1992; 2(4): 324-329.
- Yoneyama H, Hara T, Kato Y, Yamori T, Matsuura ET, Koike K. Nucleotide sequence variation is frequent in the mitochondrial DNA displacement loop region of individual human tumor cells.

Mol Cancer Res. 2005; 3(1): 14-20.

- Seyedhassani SM, Houshmand M, Kalantar SM, Modabber G, Aflatoonian A. No mitochondrial DNA deletions but more D-loop point mutations in repeated pregnancy loss. J Assist Reprod Genet. 2010; 27(11): 641-648.
- Shakhssalim N, Houshmand M, Kamalidehghan B, Faraji A, Sarhangnejad R, Dadgar S, et al. The mitochondrial C16069T polymorphism, not mitochondrial D310 (D-loop) mononucleotide sequence variations, is associated with bladder cancer. Cancer Cell Int. 2013; 13(1): 120.
- Ferreira IL, Nascimento MV, Ribeiro M, Almeida S, Cardoso SM, Grazina M, et al. Mitochondrial-dependent apoptosis in Huntington's disease human cybrids. Exp Neurol. 2010; 222(2): 243-255.
- Carew JS, Zhou Y, Albitar M, Carew JD, Keating MJ, Huang P. Mitochondrial DNA mutations in primary leukemia cells after chemotherapy: clinical significance and therapeutic implications. Leukemia. 2003; 17(8): 1437-1447.
- 20. Mousavizadeh K, Rajabi P, Alaee M, Dadgar S, Houshmand M. Usage of mitochondrial D-loop variation to predict risk for Huntington disease. Mitochondrial DNA. 2015; 26(4): 579-582.
- 21. Collins DW, Gudiseva HV, Trachtman B, Bowman AS, Sagaser A, Sankar P, et al. Association of primary open-angle glaucoma with mitochondrial variants and haplogroups common in African Americans. Mol Vis. 2016; 22: 454-471. eCollection 2016.
- Saxena R, de Bakker PI, Singer K, Mootha V, Burtt N, Hirschhorn JN, et al. Comprehensive association testing of common mitochondrial DNA variation in metabolic disease. Am J Hum Genet. 2006; 79(1): 54-61.
- Houshmand M, Larsson NG, Holme E, Oldfors A, Tulinius MH, Andersen O. Automatic sequencing of mitochondrial tRNA genes in patients with mitochondrial encephalomyopathy. Biochim Biophys Acta. 1994; 1226(1): 49-55.
- Stoneking M. Hypervariable sites in the mtDNA control region are mutational hotspots. Am J Hum Genet. 2000; 67(4): 1029-1032.
- 25. Brandon M, Baldi P, Wallace DC. Mitochondrial mutations in cancer. Oncogene. 2006; 25(34): 4647-4662.
- Huhne J, Pfeiffer H, Brinkmann B. Heteroplasmic substitutions in the mitochondrial DNA control region in mother and child samples. Int J Legal Med. 1998; 112(1): 27-30.
- 27. Bragoszewski P, Kupryjanczyk J, Bartnik E, Rachinger A, Ostrowski J. Limited clinical relevance of mitochondrial DNA mutation and gene expression analyses in ovarian cancer. BMC Cancer. 2008; 8: 292.
- Chen JZ, Gokden N, Greene GF, Mukunyadzi P, Kadlubar FF. Extensive somatic mitochondrial mutations in primary prostate cancer using laser capture microdissection. Cancer Res. 2002; 62(22): 6470-6474.
- Boles RG, Zaki EA, Lavenbarg T, Hejazi R, Foran P, Freeborn J, et al. Are pediatric and adult-onset cyclic vomiting syndrome (CVS) biologically different conditions? Relationship of adultonset CVS with the migraine and pediatric CVS-associated common mtDNA polymorphisms 16519T and 3010A. Neurogastroenterol Motil. 2009; 21(9): 936-e72.
- Van Trappen PO, Cullup T, Troke R, Swann D, Shepherd JH, Jacobs IJ, et al. Somatic mitochondrial DNA mutations in primary and metastatic ovarian cancer. Gynecol Oncol. 2007; 104(1): 129-133.
- Del Bo R, Bordoni A, Sciacco M, Di Fonzo A, Galbiati S, Crimi M, et al. Remarkable infidelity of polymerase gammaA associated with mutations in POLG1 exonuclease domain. Neurology. 2003; 61(7): 903-908.
- 32. Kenney MC, Chwa M, Atilano SR, Falatoonzadeh P, Ramirez C, Malik D, et al. Molecular and bioenergetic differences between cells with African versus European inherited mitochondrial DNA haplogroups: implications for population susceptibility to diseases. Biochim Biophys Acta. 2014; 1842(2): 208-219.
- Catelli ML, Alvarez-Iglesias V, Gomez-Carballa A, Mosquera-Miguel A, Romanini C, Borosky A, et al. The impact of modern migrations on present-day multi-ethnic Argentina as recorded on the mitochondrial DNA genome. BMC Genet. 2011; 12: 77.
- Zhang AM, Jia X, Bi R, Salas A, Li S, Xiao X, et al. Mitochondrial DNA haplogroup background affects LHON, but not suspected LHON, in Chinese patients. PLoS One. 2011; 6(11): e27750.

- Barbosa AB, da Silva LA, Azevedo DA, Balbino VQ, Mauricioda-Silva L. Mitochondrial DNA control region polymorphism in the population of Alagoas state, north-eastern Brazil. J Forensic Sci. 2008; 53(1): 142-146.
 Sukernik RI, Volodko NV, Mazunin IO, Eltsov NP, Dryomov SV,
- Sukernik RI, Volodko NV, Mazunin IO, Eltsov NP, Dryomov SV, Starikovskaya EB. Mitochondrial genome diversity in the Tubalar, Even, and Ulchi contribution to prehistory of native Siberians and their affinities to Native Americans. Am J Phys Anthropol. 2012; 148(1): 123-138.
 Paull D, Emmanuele V, Weiss KA, Treff N, Stewart L, Hua H, et
- Paull D, Emmanuele V, Weiss KA, Treff N, Stewart L, Hua H, et al. Nuclear genome transfer in human oocytes eliminates mitochondrial DNA variants. Nature. 2013; 493(7434): 632-637.
- 38. Batini C, Lopes J, Behar DM, Calafell F, Jorde LB, van der

Veen L, et al. Insights into the demographic history of African Pygmies from complete mitochondrial genomes. Mol Biol Evol. 2011; 28(2): 1099-1110.

- Loo JH, Trejaut JA, Yen JC, Chen ZS, Lee CL, Lin M. Genetic affinities between the Yami tribe people of Orchid Island and the Philippine Islanders of the Batanes archipelago. BMC Genet. 2011; 12: 21.
- Puomila A, Hämäläinen P, Kivioja S, Savontaus ML, Koivumäki S, Huoponen K, et al. Epidemiology and penetrance of Leber hereditary optic neuropathy in Finland. Eur J Hum Genet. 2007; 15(10): 1079-1089.

Fermented Garlic Extract Increases Oxygen Consumption and UCP-1 mRNA Expression in Human Adipose-Derived Stem Cells

Eulsoon Park, Ph.D.¹, Seung-Hwa Baek, Ph.D.², Keuk-Su Bang, Ph.D.³, Na-Hyung Kim, Ph.D.⁴,

Koichi Takimoto, Ph.D.1*

1. Department of Bioengineering, Nagaoka University of Technology, Nagaoka, Niigata, Japan

2. Department of Biofood Science and Biotechnology, Chungbuk Provincial University, Okcheon-gun, Chungbuk, Republic of Korea 3. Department of Oriental Medicine Resources, College of Environmental and Bioresource Science, Chonbuk National University, Ik-san

City, Chonbuk, Republic of Korea

4. Research Institute of HtO Life, HtO Life Co., LTD, Wanju-gun, Republic of Korea

*Corresponding Address: Department of Bioengineering, Nagaoka University of Technology Kamitomioka, Nagaoka, Niigata, Japan

Email: koichi@vos.nagaokaut.ac.jp

Received: 14/September/2018, Accepted: 19/November/2018

Abstract

Fermented garlic, often called black garlic, is a traditional food ingredient used in Asian cuisine and possesses various health benefits including anti-obesity activity. The anti-obesity effects of fermented garlic might, in part, might be mediated through direct actions of its components on adipocytes. To test this hypothesis, we examined whether fermented garlic extract might stimulate the metabolic activity of human adipose-derived stem cells (ADSCs) in culture. Cell viability measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay exhibited a complex dose-response relationship. The lowest concentration (0.4 mg/ml) reduced cell viability (P<0.05 compared to no extract, Bonferroni's multiple comparison), whereas higher concentrations (0.8 and 1.0 mg/ml) resulted in higher cell viability (P<0.05 as compared to 0.4 mg/ml). However, the extract at concentrations >2 mg/ml markedly decreased cell viability. Higher cell viability observed following treatment with 0.8~1.0 mg/ml might be associated with raised oxygen consumption. Fluorescent dye-based measurement revealed that the garlic extract at 1.0 mg/ml significantly increased oxygen consumption. We also detected a significant increase in mRNA expression levels of uncoupling protein-1 (UCP-1). These findings suggest that fermented garlic stimulates the basal metabolic activity of human ADSCs.

Keywords: Adipose-Derived Stem Cells, Garlic, Mitochondrial Uncoupling Protein, Oxygen Consumption, Thermogenesism

Cell Journal(Yakhteh), Vol 21, No 3, October-December (Autumn) 2019, Pages: 357-362 .

Citation: Park E, Baek SH, Bang KS, Kim NH, Takimoto K. Fermented garlic extract increases oxygen consumption and UCP-1 mRNA expression in human adipose-derived stem cells. Cell J. 2019; 21(3): 357-362. doi: 10.22074/cellj.2019.6382.

Black garlic is produced by fermentation under high temperature and humidity. As it possesses unique texture and flavor and lacks offensive smells, it has become a popular ingredient not only in Asian cuisines, but for other cooking styles of the world. In addition, black garlic has been shown to produce various health benefits, some of which may not be seen with fresh product (1, 2). For example, dietary consumption of black garlic decreased body weight and fat accumulation in a rodent model of obesity (3, 4). Similarly, methanol extract of black garlic reduced fat masses and altered expression of various genes involved in lipid metabolism (5). Notably, the abovementioned changes were observed in the absence of any decline in total food consumption. Therefore, components of black garlic may stimulate basal metabolic activity in vivo.

Nonshivering thermogenesis accounts at least in part for alterations in the basal metabolic rate under physiological conditions. Brown adipose tissues are primarily responsible for nonshivering thermogenesis in rodents and human infants. Studies conducted during the last ten years have established that adult humans also possess active brown adipose tissues (6-8). Furthermore, cells present in white adipose tissues of adult humans become thermogenic under certain conditions (9-11). Considering the alarming increase in the prevalence of obesity and its associated diseases, browning of white fats has gained much attention as a possible therapeutic approach against these detrimental conditions.

Known *in vivo* health benefits of black garlic consumption are likely to influence various organs and complex physiological mechanisms. It is also possible that black garlic possibly exerts direct beneficial actions on adipocytes. To test this hypothesis, we used human adipose-derived stem cells (ADSCs) and fermented garlic, a type of black garlic that is prepared under sterile and controlled conditions (12). In this study, we found that fermented garlic extract stimulates the basal metabolic activity and browning of human ADSCs.

Ordinary black garlic used for culinary purposes is prepared by fermenting unpeeled garlic using supplemented or naturally-occurring microorganisms. The preparing procedure significantly differs from one region to another and various microorganisms present in the producing environment may potentially affect the final product. Thus, we used fermented garlic prepared under conditions with minimized contamination caused by environmental microorganisms from HtO Life Co., Ltd. (Wanju-gun, Republic of Korea). This product was prepared by fermentation of peeled and crushed garlic cloves. Briefly, peeled garlic cloves were sterilized using ozonized water and crushed into a paste-like preparation. The garlic paste was then fermented using the strain, Bacillus subtilis subsp. subtilis KACC91554P, under aerobic conditions. Water-soluble extract was collected by ultrafiltration, and hot air-dried using a spray dryer (Eyela SD-1000, Eyela, Japan). The prepared fermented garlic was tested for various components at Namhae Garlic Research Institute (Namhae, Republic of Korea) to ensure the consistency of the preparations (12).

The dried fermented garlic extract was re-dissolved in water at a final concentration of 100 mg/ml by rigorous vortexing and sonication. Undissolved materials were eliminated by centrifugation, followed by filtration through a cellulose acetate membrane (pore size 0.2-mm). The concentrations of fermented garlic mentioned in this paper indicate those measured based on the initial dried powder without considering the undissolved and eliminated materials.

Human adipose-derived stem cells (ADSCs) were purchased from Cellular Engineering Technologies (Coralville, IA, USA). Cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (Gibco, New Zealand), 50 U/ml penicillin and 50 μ g/ml streptomycin (Nacalai Tesque, Japan) under 5% CO₂ atmosphere at 37°C.

Human ADSCs were seeded in a 96-well dish at ~5000 cells/well. Two days after seeding, cells were treated with fermented garlic extract or water for additional

2 days. Cell viability was then determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) according to the manufacture's protocol (Thermo Fisher Scientific, Walthum, MA, USA).

Total cellular oxygen consumption was determined by measuring extracellular molecular oxygen using a phosphorescent oxygen-sensitive dye (Abcam, UK). Human ADSCs were treated with 1.0 mg/ml fermented garlic extract or water (1/100 volume) for 2 days. Cells were then subjected to oxygen consumption assays according to the manufacturer's protocol. Fluorescence was monitored every 1.5 minutes over 90 minutes at 37°C by a plate reader (Tecan, Switzerland). A linear portion of blank-collected fluorescent intensity in each sample was used to estimate the oxygen consumption rate. Antimycin A, an inhibitor of the electron transport chain, almost completely eliminated the time-dependent increase in fluorescent intensity.

Total RNAs were isolated from cultured human ADSCs using a phenol-based reagent (Sepasol, Nacalai Tesque, Japan) and their concentrations were estimated using absorbance at 260 nm=40 ng/ml. First-strand cDNAs were synthesized using 0.1 mg total RNA with a mixture of oligo(dT) and random primers (ReverTra Ace Master Mix, Japan). PCR was carried out using synthesized cDNA sample (0.1 to 1.0 ml) using primers (Table 1) under the following conditions: denaturation at 95°C for 5 seconds, annealing at 58°C for 5 seconds, and extension at 72°C for 1 minute for 24~30 cycles. Sample volume and cycle numbers were varied for different primer sets and samples. PCR products were separated by a 1.2% agarose gel and stained with ethidium bromide. We semi-quantitatively estimated relative mRNA levels by measuring ethidium bromidestained band intensities using a CCD camera-based imaging system (UVP, Upland, CA).

Table 1: Primers used in this study								
Name	Primer sequence (5'-3')	Position	Length	GenBank accession				
UCP-1	F: AGGAGTGGCAGTATTCATTGG	448-686	239	NM_021833				
	R: TCACAAAGGCCTCCTTCATTAG							
PPARGC1A	F: CAGCTCCAAGACCAGGAAAT	1474-1696	223	NM_013261				
	R: CCCAAGGGTAGCTCAGTTTATC							
PPARG	F: GCTGGCCTCCTTGATGAATAA	1234-1438	205	NM 138712				
	R: GCGGTCTCCACTGAGAATAATG			_				
	E. CTCA ACCCATTTCCTCCTATTC	124 262	120	NM 002046				
GAPDH		124-202	139	NWI_002046				
	R: CATGGGTGGAATCATATTGGAA							

Fermented garlic contains various chemicals that may influence cellular metabolism in distinct ways. We first used MTT assay which is based on cellular metabolic activity in terms of reducing MTT. The MTT assay results should represent total cellular metabolic activity or cell viability in the sample. These assays are widely used to measure cell proliferation and/or chemical's toxicity. We treated human ADSCs with fermented garlic extract at various concentrations for 2 days and determined cell viability (Fig.1A). The cell viability showed a complex dose-response relationship. At concentrations as low as 0.4 mg/ml, the extract reduced cell viability. However, the extract at slightly higher concentrations, 0.8 and 1.0 mg/ml, caused a regain in the viability. Further increasing the concentration to >2 mg/ml resulted in marked reductions in the viability. Since black garlic extract might influence cell growth or induce cell toxicity, we also determined live cell numbers and percentages using trypan blue exclusion following treatment with several concentrations of the extract (Fig.1B). No significant changes in the live cell number or percentages were detected at the extract concentrations up to 1.0 mg/ml (live cell percentages are shown in Figure 1B, data not shown for live cell numbers). At the highest concentration (4.0 mg/ml) in the current study, the live cell number and percentage slightly decreased (less than 10% of total cells). Thus, the observed rise in cell viability at concentrations around 1.0 mg/ml may be due to the presence of a component in fermented garlic extract that enhances metabolic activity of human ADSCs.

We wished to further corroborate the possibility that fermented garlic extract stimulates the metabolic activity of ADSCs. Since MTT assays may be associated with off-target effects, we measured cellular oxygen consumption using a molecular oxygensensitive dye (Fig.2). Cellular oxygen consumption was significantly higher in cells treated with 1.0 mg/ ml of the extract for 2 days than vehicle (water)-treated cells. Given that the treatment with fermented garlic extract at this concentration did not influence live cell number or percentage, these results further support the possibility that the extract enhances the metabolic activity of ADSCs.

Then we examined whether the observed increase in oxygen consumption is associated with the browning of human ADSCs. We measured mitochondrial uncoupling protein-1 (*UCP-1*) mRNA levels; *UCP-1* is a key protein in proton leak from the mitochondrial inner membrane and physiological heat generation (Fig.3A, B). Low levels of *UCP-1* mRNA were detected in most samples that were not treated with fermented garlic extract. Importantly, *UCP-1* mRNA level markedly increased following treatment with 1.0 or 2.0 mg/ml of the extract for 2 days. Thus, the *UCP-1*-based proton leak may in part mediate the fermented garlic extract-induced increase in metabolic activity of human ADSCs. We also tested whether fermented garlic might increase mRNA levels for peroxisome proliferator-activated receptor- γ (*PPARG*) and its coactivator-1 α (*PPARGC1A*), master regulators of the *UCP-1* gene transcription and mitochondrial biogenesis (Fig.3C). Treatment with fermented 1.0 mg/ml garlic extract for 2 days raised *PPARG* and *PPARGC1A* mRNA expression. These findings suggest that fermented garlic induces the browning of human ADSCs.

Α

В









Fig.2: Fermented garlic extract increases cellular oxygen consumption of human adipose-derived stem cells (ADSCs). Human ADSCs were treated with 1.0 mg/ml fermented garlic extract (garlic) or vehicle (1/100 volume of water, none) for 2 days. Oxygen consumption was determined by measuring changes in molecular oxygen in the culture medium with a phosphorescent dye (n=8 from three independent cell preparations). **; P<0.01 shows significant differences as compared to none (t test was used for data analysis).

Fermented or black garlic has been shown to produce various health benefits. In particular, intake of black garlic reduces body weight and fat masses, and normalizes physiological and biochemical parameters in animal models of obesity (3-5). However, it remains unknown whether component(s) in these fermented garlic products act directly on adipocytes to produce any beneficial changes. In this paper, we showed that fermented garlic extract increases oxygen consumption and UCP-1 mRNA level in cultured human ADSCs. In addition, the extract increased mRNA levels of *PPARy* and *PGC-1* α that play pivotal roles in *UCP-1* expression and mitochondrial biogenesis in adipocytes (13). Thus, component(s) present in fermented garlic may directly activate thermogenesis of these adult body-residential cells.

It has become evident that cells located in white adipose tissues of adult rodents (14-17) and humans (9-11) become thermogenic under certain conditions. Moreover, prolonged cold exposure or treatment with b3 agonists converts white fats to brown fat-like heatgenerating tissues in intact animals (18, 19). These brown adipocyte-like cells possess gene expression profiles that are distinct from those of standard brown adipocytes and are called "brite" or "beige" adipocytes (20, 21). The overweight population has tripled in the last 40 years in the world; also, obesity has appeared as a major risk factor for various diseases. Therefore, inducing browning of white adipose tissue-residential cells is considered to hold a promising therapeutic potential against this major health problem. Our finding which showed that fermented garlic, a simple food ingredient, can stimulate this process, may provide basis for economical interventions for the prevention

of obesity. Additionally, fermented or black garlic is known to enhance food flavor, and may be easily introduced to diverse cooking styles.



Fig.3: Fermented garlic extract increases expression of *UCP1* and brown adipocyte marker mRNAs. Human adipose-derived stem cells (ADSCs) were treated with fermented garlic extract at indicated concentrations for 2 days. Reverse transcription-polymerase chain reaction (RT-PCR) was performed with primers for *UCP1*, *PPARGC1A*, *PPARG* or *GAPDH*. **A**. Representative PCR results for *UCP-1* mRNA expression, **B**. *UCP-1* mRNA levels were semi-quantitatively estimated using *GAPDH* mRNA level as an internal control (n=3 from three independent cell preparations). **; P<0.01 shows significant differences as compared to 0 mg/ml (one sample test), and **C**. Human ADSCs were treated with 0.1 mg/ml fermented garlic extract (F. Garlic) or water (vehicle) for 2 days. Representative PCR data show increased expression of *PPARGC1A* and *PPARG* mRNAs, as well as *UCP1* transcript.

Fermented or black garlic contains various chemical components. Unlike fresh garlic, the sulfur-containing alliin and its converted substances with offensive flavors are much less abundant in fermented products (1, 2, 22). In contrast, the main sulfur-containing product appears to be S-allylcysteine in aged or fermented garlic products. In addition, polyphenols, flavonoids, and several compounds generated by the Amadori and Heyns rearrangements are found at much higher levels in these garlic products (1, 2, 23, 24). Some of these compounds were shown to possess biological activities. For example, S-allylcysteine possesses antioxidant and anti-inflammatory activities (25). Likewise, polyphenols in aged or fermented garlic are proposed to contribute to antioxidant properties of these products (26). In this study, we used total water extract of fermented garlic. This preparation yielded a complex dose-response change in MTT assays, likely due to the presence of various components and their potential interactions. Thus, it would be certainly important to identify component(s) present in this preparation that mediate(s) the observed stimulation of oxygen consumption and UCP-1 mRNA expression. Taken together, further identification of the chemical component(s) responsible for the observed effects, as well as molecular mechanistic studies, may yield novel and useful information on the use of fermented garlic for prevention and treatment of obesity and its associated diseases.

Acknowledgements

This research did not receive any specific grant from funding agencies of the public, commercial, or notfor-profit sectors. Na-Hyung Kim is an employee of the HtO Life Co., LTD, a manufacturer of fermented garlic products, and prepared fermented garlic extract for the study described in this manuscript. The authors declare no other conflicting interests associated with the manuscript.

Authors' Contributions

E.P., K.T.; Designed the present study, performed experiments using adipose-derived stem cells, evaluated and statistically analyzed the data, and prepared the manuscript. S.-H.B., K.-S.B., N.-H.K.; Perceived original idea of the present study and participated in study design. N.-H.K.; Prepared and quality-tested fermented garlic extract. All authors read and approved the final manuscript.

References

- Martins N, Petropoulos N, Ferreira IC. Chemical composition and bioactive compounds of garlic (Allium sativum L.) as affected by pre- and post-harvest conditions: a review. Food Chem. 2016; 211: 41-50.
- 2. Kimura S, Tung YC, Pan MH, Su NW, Lai YJ, Cheng KC. Black

garlic: A critical review of its production, bioactivity, and application. J Food Drug Anal. 2017; 25(1): 62-70.

- Jung YM, Lee SH, Lee DS, You MJ, Chung IK, Cheon WH, Kwon YS, Lee YJ, Ku SK. Fermented garlic protects diabetic, obese mice when fed a high-fat diet by antioxidant effects. Nutr Res. 2011; 31(5): 387-396.
- Ha AW, Ying T, Kim WK. The effects of black garlic (Allium satvium) extracts on lipid metabolism in rats fed a high fat diet. Nutr Res Pract. 2015; 9(1): 30-36.
- Chen YC, Kao TH, Tseng CY, Chang WT, Hsu CL. Methanolic extract of black garlic ameliorates diet-induced obesity via regulating adipogenesis, adipokine biosynthesis, and lipolysis. J Functional Foods. 2014; 9: 98-108.
- Cypess AM, Lehman S, Williams G, Tal I, Rodman D, Goldfine AB, Kuo FC, Palmer EL, Tseng YH, Doria A, Kolodny GM, Kahn CR. Identification and importance of brown adipose tissue in adult humans. N Engl J Med. 2009; 360(15): 1509-1517.
- Lee P, Zhao JT, Swarbrick MM, Gracie G, Bova R, Greenfield JR, Freund J, Ho KK. High prevalence of brown adipose tissue in adult humans. J Clin Endocrinol Metab. 2011; 96(8): 2450-2455.
- Virtanen KA, Lidell ME, Orava J, Heglind M, Westergren R, Niemi T, Taittonen M, Laine J, Savisto NJ, Enerback S, Nuutila P. Functional brown adipose tissue in healthy adults. N Engl J Med. 2009; 360(15): 1518-1525.
- Tiraby C, Tavernier G, Lefort C, Larrouy D, Bouillaud F, Ricquier D, Langin D. Acquirement of brown fat cell features by human white adipocytes. J Biol Chem. 2003; 278(35): 33370-33376.
- Elabd C, Chiellini C, Carmona M, Galitzky J, Cochet O, Petersen R, Penicaud L, Kristiansen K, Bouloumie A, Casteilla L, Dani C, Ailhaud G, Amri EZ. Human multipotent adipose-derived stem cells differentiate into functional brown adipocytes. Stem Cells. 2009; 27(11): 2753-2760.
- Carrière A, Jeanson Y, Berger-Müller S, André M, Chenouard V, Arnaud E, Barreau C, Walther R, Galinier A, Wdziekonski B, Villageois P, Louche K, Collas P, Moro C, Dani C, Villarroya F, Casteilla L. Browning of white adipose cells by intermediate metabolites: an adaptive mechanism to alleviate redox pressure. Diabetes. 2014; 63(10): 3253-3265.
- Jung ES, Park SH, Choi EK, Ryu BH, Park BH, Kim DS, Kim YG, Chae SW. Reduction of blood lipid parameters by a 12-wk supplementation of aged black garlic: a randomized controlled trial. Nutrition. 2014; 30(9): 1034-1039.
- Guerra C, Koza RA, Yamashita H, Walsh K, Kozak LP. Emergence of brown adipocytes in white fat in mice is under genetic control. Effects on body weight and adiposity. J Clin Invest. 1998; 102(2): 412-420.
- Puigserver P, Wu Z, Park CW, Graves R, Wright M, Spiegelman BM. A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. Cell. 1998; 92(6): 829-39.
- Koza RA, Hohmann SM, Guerra C, Rossmeisl M, Kozak LP. Synergistic gene interactions control the induction of the mitochondrial uncoupling protein (Ucp1) gene in white fat tissue. J Biol Chem. 2000; 275(44): 34486-34492.
- Coulter AA, Bearden CM, Liu X, Koza RA, Kozak LP. Dietary fat interacts with QTLs controlling induction of Pgc-1 alpha and Ucp1 during conversion of white to brown fat. Physiol Genomics. 2003; 14(2): 139-147.
- Xue B, Coulter A, Rim JS, Koza RA, Kozak LP. Transcriptional synergy and the regulation of Ucp1 during brown adipocyte induction in white fat depots. Mol Cell Biol. 2005; 25(18): 8311-8322.
- Loncar D. Convertible adipose tissue in mice. Cell Tissue Res. 1991; 266(1): 149-161.
- Cousin B, Cinti S, Morroni M, Raimbault S, Ricquier D, Penicaud L, Casteilla L. Occurrence of brown adipocytes in rat white adipose tissue: molecular and morphological characterization. J Cell Sci. 1992; 103(Pt4): 931-942.
- Wu J, Cohen P, Spiegelman BM. Adaptive thermogenesis in adipocytes: is beige the new brown? Genes Dev. 2013; 27(3): 234-250.
- 21. Chen Y, Pan R, Pfeifer A. Fat tissues, the brite and the dark sides. Pflugers Arch. 2016; 468(11): 1803-1807.
- 22. Bae SE, Cho SY, Won YD, Lee SH, Park HJ. Changes in S-allyl

cysteine contents and physicochemical properties of black garlic during heat treatment. Food Science Technol. 2014; 55(1): 397-402.

- 23. Yuan H, Sun L, Chen M, Wang J. The comparison of the contents of sugar, Amadori, and Heyns Compounds in fresh and black garlic. J Food Sci. 2016; 81(7): C1662-C1668.
- 24. Lu X, Li N, Qiao X, Qiu Z, Liu P. Composition analysis and antioxidant properties of black garlic extract. J Food Drug Anal.

2017;25:340-349.

- Colín-González AL, Ali SF, Túnez I, Santamaría A. On the antioxidant, neuroprotective and anti-inflammatory properties of S-allyl cysteine: an update. Neurochem Int. 2015; 89(2): 83-91.
- Nencini C, Menchiari A, Franchi GG, Micheli L. In vitro antioxidant activity of aged extracts of some Italian Allium species. Plant Foods Hum Nutr. 2011; 66(1): 11-16.