

Alginate Microcapsules as Nutrient Suppliers: An *In Vitro* Study

Ahad Khoshzaban, M.Sc., D.D.S., Ph.D.^{1,2,3}, Peyman Keyhanvar, Ph.D.², Elham Delrish, M.Sc.²,
Farhood Najafi, Ph.D.⁴, Saeed Heidari Keshel, Ph.D.², Ikuya Watanabe, Ph.D.⁵,
Alireza Valanezhad, Ph.D.⁵, Tahereh Jafarzadeh Kashi, Ph.D.^{1,3*}

1. Iranian Tissue Bank and Research Center, Imam Khomeini Medical Complex Hospital, Tehran University of Medical Sciences, Tehran, Iran
2. Stem Cell Preparation Unit, Farabi Eye Hospital, Tehran University of Medical Sciences, Tehran, Iran
3. Department of Dental Biomaterials, School of Dentistry, Tehran University of Medical Sciences, Tehran, Iran
4. Department of Resin and Additives, Institute for Color Science and Technology, Tehran, Iran
5. Department of Bio-Engineering, Nagasaki University, Nagasaki, Japan

*Corresponding Address: P.O. BOX: 14395-43, End of North Karegar, Department of Dental Biomaterials, School of Dentistry, Tehran University of Medical Sciences, Tehran, Iran
Email: jafarzat@tums.ac.ir

Received: 9/Aug/2016, Accepted: 20/Jan/2017

Abstract

Objective: Alginate, known as a group of anionic polysaccharides extracted from seaweeds, has attracted the attention of researchers because of its biocompatibility and degradability properties. Alginate has shown beneficial effects on wound healing as it has similar function as extracellular matrix. Alginate microcapsules (AM) that are used in tissue engineering as well as Dulbecco's modified Eagle's medium (DMEM) contain nutrients required for cell viability. The purpose of this research was introducing AM in medium and nutrient reagent cells and making a comparison with control group cells that have been normally cultured in long term.

Materials and Methods: In this experimental study, AM were shaped in distilled water, it was dropped at 5 mL/hours through a flat 25G5/8 sterile needle into a crosslinking bath containing 0.1 M calcium chloride to produce calcium alginate microspheres. Then, the size of microcapsules (300-350 μm) were confirmed by Scanning Electron Microscopy (SEM) images after the filtration for selection of the best size. Next, DMEM was injected into AM. Afterward, adipose-derived mesenchymal stem cells (ADSCs) and Ringer's serum were added. Then, MTT and DAPI assays were used for cell viability and nucleus staining, respectively. Also, morphology of microcapsules was determined under invert microscopy.

Results: Evaluation of the cells performed for spatial media/microcapsules at the volume of 40 μl , showed ADSCs after 1-day cell culture. Also, MTT assay results showed a significant difference in the viability of sustained-release media injected to microcapsules ($P < 0.05$). DAPI staining revealed living cells on the microcapsules after 1 to 7-day cell culture.

Conclusion: According to the results, AM had a positive effect on cell viability in scaffolds and tissue engineering and provide nutrients needed in cell therapy.

Keywords: Alginate, Cell Culture, Cell Viability, Growth Media, Microcapsule

Cell Journal (Yakhteh), Vol 20, No 1, Apr-Jun (Spring) 2018, Pages: 25-30

Citation: Khoshzaban A, Keyhanvar P, Delrish E, Najafi F, Heidari Keshel S, Watanabe I, Valanezhad A, Jafarzadeh Kashi T. Alginate microcapsules as nutrient suppliers: an in vitro study. Cell J. 2018; 20(1): 25-30. doi: 10.22074/cellj.2018.4508.

Introduction

Alginate is a popular group of anionic polysaccharides extracted from seaweeds (1, 2) that could be produced by some brown algae and certain bacteria species such as *Azotobacter vinelandii* and *Pseudomonas aeruginosa* (2). Also, alginate has different applications and could be found in nature in the forms heteropolysaccharide hydrogel of β -D-mannuronate and α -L-guluronate, physically cross-linked with divalent ions such as calcium to form an anionic hydrogel (1, 3, 4). The simple, mild aqueous-based gel formation of sodium alginate in the presence of divalent cations is suitable for encapsulation of various drugs with different properties (3).

The scaffold usage is justified as it provides a suitable headstock for cell growth, proliferation and differentiation (2). *In vitro* and *in vivo* studies revealed that no scaffold can guarantee long-term viability of cells. Because of

biocompatible, non-toxic and non-immunogenic properties of alginate, it is used as a common scaffold which functions both as a cytokines carrier and scaffold (4). In addition, it can be used in capsule form as a carrier of the stem cells (3). One of the applications of this material is protection of stem cells from immune responses (4). Therefore, the nutrient release and cytokines properties of alginate can be influenced its clinical application particularly in bone tissue engineering and vascularization (2, 3).

Alginate microcapsules (AM) can be saturated with different solutions (3) and they may have direct effects on cells. It is reported that AM helps the smart differentiation of stem cells and induced pluripotent stem (IPS) cells. It is reported that alginate can be used for stem cells encapsulation (5).

The microcapsules have disadvantages such as being

easily ruptured as they possess low micromechanical properties. Up to the present, several studies have been done on application of different materials or changing the synthesis protocols to improve the micromechanical properties of AM (4). For instance, CaCl₂-anionic hydrogel-chitosan, G (α -L-guluronic acid) and M (β -D-mannuronic acid) were used to enhance the micromechanical properties of AM (1, 3).

The molecular composition of alginates depends on the organism and isolated tissue by which the alginate has been produced (3). Alginates prepared from the stipes of old *L. Hyperborea* kelp contain the highest content of α -L-guluronic acid residues while alginate from *Ascophyllum nodosum* and *Lonicera japonica*, has lower amounts of α -L-guluronic acid (6). It is revealed that alginate is not subjected to a regular repeatability according to Bernoullian statistics (i.e. the units of alginate are found in a separate chain and it has monomers along) (3). In cell therapy, the optimum time within which cells should be viable is at least 72 hours and alginate is expected to have the capacity of maintaining cell viability for this period (7). In most studies, AM is used for stem cell maintenance and differentiation (5-7). Also, alginate hydrogel accelerates wound healing process and prevents infection (8).

According to AM medical activity or alginate hydrogel, the supply of nutrient solution has been used in cell culture (8). Nutrients (cell culture media) have been frequently used in cell culture but clinical investigations indicated that cells injection might not work in the absence of nutrients (7). The main objective of the present study is application of microcapsules as suppliers of nutrients for growth and proliferation of cells in comparison with conventional culture method.

Materials and Methods

Alginate microcapsules synthesis

In this experimental study, sodium alginate, potassium and calcium chlorides were procured from Sigma Aldrich, UK. The chemicals were used without further purification. The particle size and surface morphology of

the microcapsules were examined by Scanning Electron Microscopy (SEM) (Fig.1A, B).

Fabrication of microspheres

The AM were typically synthesized as previously described (6). A total of 3.1 g of alginate was added to 100 mL of distilled water and the resultant mixture was stirred until the alginate was completely dissolved. Afterward, the alginate solution was filtered and passed through a syringe pump to form droplets. Then, it was dropped at 5 mL/hour through a flat 25G5/8 sterile needle into a crosslinking bath containing 0.1 M calcium chloride to produce calcium alginate microspheres (6-8). To reduce droplets size, airflow of 12.5 L/minute through 3 mm tube was used over the needle. The alginate droplets were transformed to alginate beads by gelling in a 100 mM CaCl₂ solution (containing 2 mM KCl) for at least 10 minutes. Subsequently, the beads were washed three times in Ca²⁺, Mg²⁺-free solution (GibcoBRL) (pH=7.0) containing 150 mM of NaCl (Merelbeke, Belgium). The capsules were suspended for another 3 minutes in a 0.3% solution of alginate. As a result, the capsules had a diameter of 300 μ m (Fig.1A).

Particle size test

For selection of the best size of microcapsules, diffraction particle size analyzer (0.03-1000 μ m) SALD-2201 laser (Shimadzu, Japan) was used. According to ISO 13320 (9) and USP (10), this method is suitable for micron-sized polymer capsules such as alginate. The obtained graph showed size variation and filtration which was used to select the best size.

Nutrient load in the microcapsules

After synthesis of microcapsules, they were transferred and pictures were taken under inverted florescent microscope (CTI, Spain) (Fig.1C). Then, 10 μ L of the nutrient media containing DMEM low glucose (Gibco, USA) and 10% fetal bovine serum (FBS, Gibco, USA) was loaded by super fine needles (Aesculap, Germany) (Fig.2).

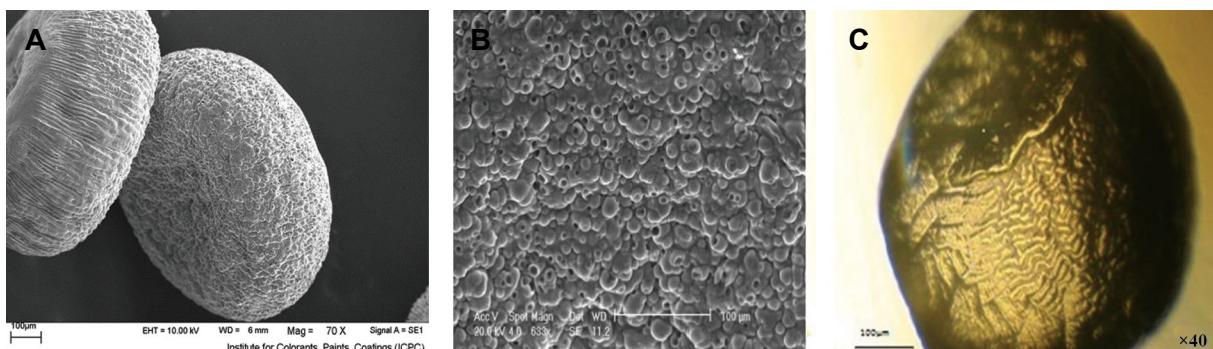


Fig.1: Alginate microcapsule (AM) scanning electronic and microscopic views. **A.** Two AM in scanning electron microscopy (SEM) view ($\times 70$), **B.** AM view in SEM view (20 KV), and **C.** One microcapsule under invert microscope ($\times 40$).

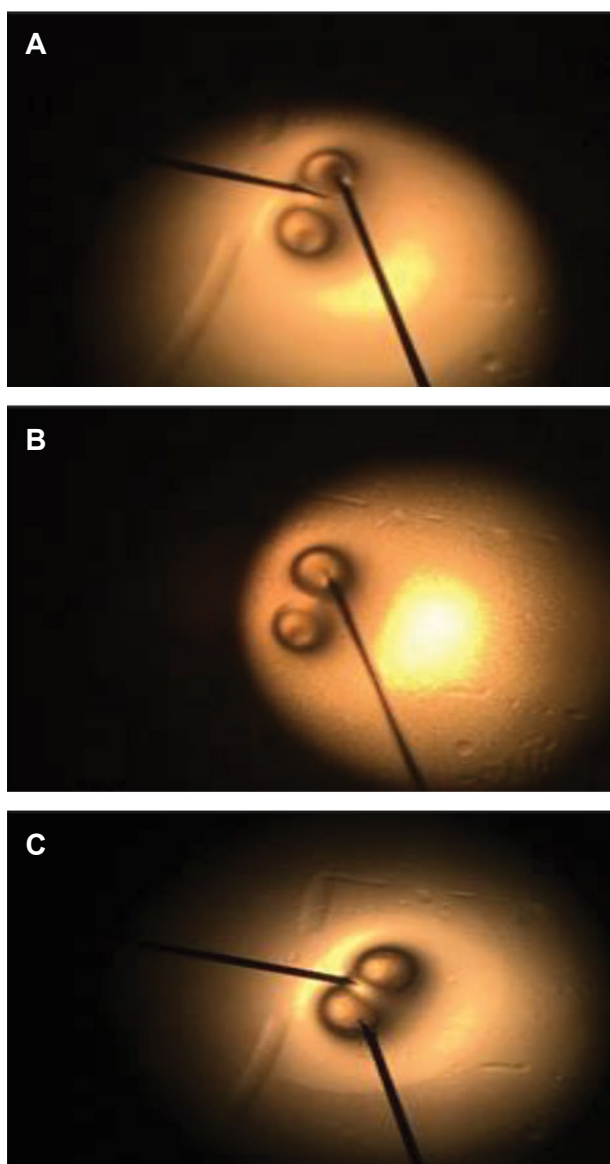


Fig.2: Injection media into microcapsules. **A.** Penetration of needle (32 G) in alginate microcapsule (AM), **B.** Filling procedure, and **C.** AM filled with nutrient (DMEM).

Releasing test

Micro-capsules were weighed before and after injection of cell culture media (on day 1, 2 and 10). Microcapsules' weight was equivalent to the weight of the empty microcapsules on day 10.

Adipose-derived mesenchymal stem cell isolation, identification and culture

Adipose-derived mesenchymal stem cells (ADSCs) were isolated by enzymatic digestion according to Park AM protocol. Briefly, lipoaspirate tissues (25 ml) were taken from two volunteers and washed with PBS in a 50-ml Falcon tube. The tissues were then digested with an equal volume of 0.2% collagenase type IV at 37°C for 15 minutes, and the stromal vascular fraction cells were isolated by centrifugation at 300 g at room temperature. Viable cells (1×10^6) were cultured in 75-cm² flasks

in 10% FBS-supplemented medium (DMEM/F 12). After 2 days, the unattached cells were discarded by replacing the medium with fresh medium. The medium was subsequently changed twice a week. At 80 to 90% confluence, both types of cells were harvested with trypsin-ethylene diamine tetra acetic acid (EDTA, Gibco, UK) and subsequently replated at 2000 cells/cm². For analysis of surface markers expression, at passage 3, AMSCs were washed three times with phosphate-buffered saline (PBS), then incubated with a blocking solution of 3% serum in PBS for 30 minutes. After centrifugation, 5×10^5 cells were suspended in blocking solution, then incubated with antibodies against human CD31, CD166, CD90, CD44, CD73, CD34 and CD 45 (Abcam, UK). After incubation for 30 minutes, the cells were washed with PBS, then analysis was made by using FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) according to the standard procedures (Figs.3, 4).

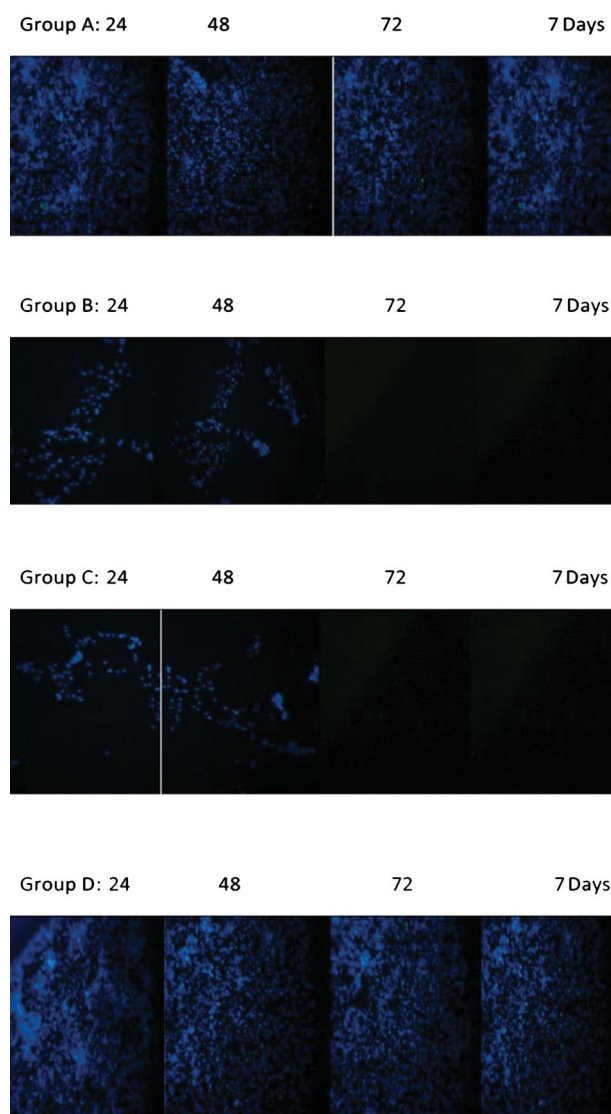


Fig.3: DAPI staining cells at different time points in 4 groups. In groups B and C dark fields were observed after 72 hours and 7 days (CTI Microscope, Spain, $\times 20$).

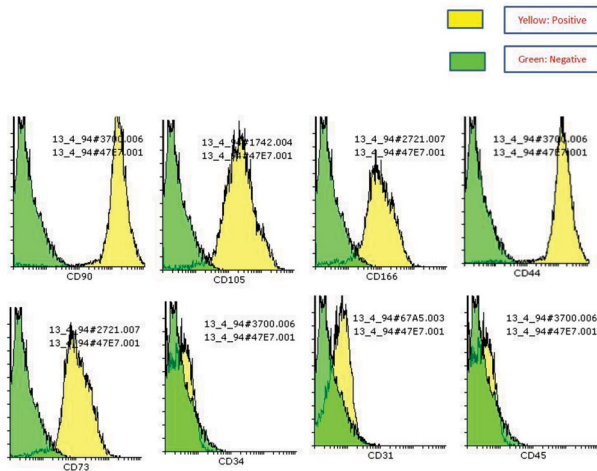


Fig.4: Adipose-derived mesenchymal stem cells flow cytometry.

Viability cells adhering by microcapsules and nutrient

After loading the microcapsules, the experimental cells (Human Adipose-Derived Mesenchymal Stem Cells, AMS) were used in Stem Cell Preparation Unit Lab, Farabi Eye Hospital, Tehran, Iran. All information concerning this cell line was recorded in Farabi Eye Hospital.

Experimental and control groups

The cells were cultured 96-well plates (Falcon, USA), at the volume of 40 μ l. Four experimental groups were used to determine the role of AM in cell contact and viability in cell therapy as compared to nutrient media. Group A (positive control): AMS cells treated with standard nutrient media 40 μ l+20 μ l [DMEM low glucose and 10% FBS Gibco, USA]. Group B (experimental control): AMS cells (40 μ l) with Ringer's serum (20 μ l, Razi Co, Iran). Group C: AMS cells (40 μ l) with empty AM+Ringer's serum. Group D: AMS cells (40 μ l) with AM+standard nutrient media+Ringer's serum (20 μ l).

MTT analysis

Viability of AMSCs grown on AM was assessed by using 3,4,5 dimethylthiazol-2-yl-2,5 diphenyl tetrazolium bromide (MTT) assay according to the protocol of MTT. Cells that were grown in normal media without AM served as a control for the interpretation of data. Then, 4 wells were selected from 96 wells for each group. Totally from 4 groups, 16 wells were filled. For each group 20 μ l of MTT solution was added, then the plates were transferred to a CO₂ incubator (Sanyo, Japan). After 4 hours, the liquid was removed and 100 μ l dimethyl sulfoxide (DMSO) was added. Finally, each of 96-well was transferred to the ELISA reader (Bio-Rad, USA). Then, the plates were read at 570 nm and the reference optical density (OD) was 600 nm. For each plate, MTT assay was done at 24, 48, and 72 hours and on day 7.

DAPI staining

The DAPI assay was done based on the following steps. 4', 6' diamino-2-phenylindole 2HCl was used for specific staining at pH=7. In order to stain, each well was fixed using 60 μ l paraformaldehyde (PFA) 4% for 8 minutes. The PFA should be dumped onto paper towels and wrapped in aluminum foils before disposal. Then, cells were washed with 60 μ l/well of 1X PBS, for 3 times each time for 5 minutes. Cells were mobilized using 60 μ l/well of 0.1% Triton-X-100 for 10 minutes and this process was repeated for 3 times. Cells were stained with 50 μ l/well of DAPI (1:2000 dilution, in 1X TBST) for 5 minutes and 50 μ l/well of 1X PBS was added to keep the cells hydrated while imaging on the Image Express Micro (Fig.3).

Statistical analysis

The results are presented as mean \pm SD. The statistical differences were analyzed by one-way ANOVA followed by Dunnett's tests. A P<0.05 was considered to be significant. All assays were performed in quadruplicate.

Results

MTT analysis

Group A had a cell viability of 95% \pm 3 at 24, 48, and 72 hours and on day 7. In groups B and C, after 24 hours, the number of viable cells reduced; therefore, after day 7, particularly viable cells were not seen (viable cells or carcass were 0% \pm 3). Group D showed the same results as group A. Accordingly, there were no significant differences between groups A and D (P>0.05). A significant difference was observed in groups B and C compared to group D (P<0.05, Fig.5).

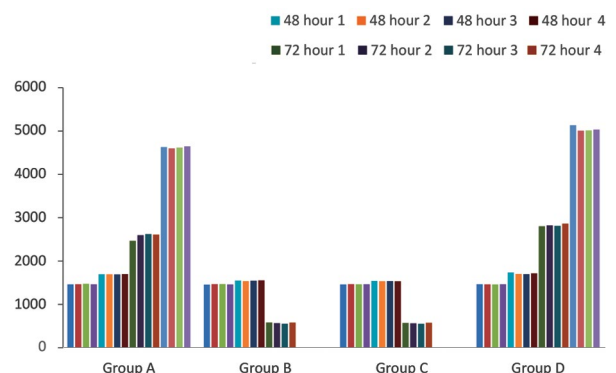


Fig.5: MTT Assay results for each group (4 wells per group). Group B and C had no viable cells after 7 days.

Particle size analysis

Microcapsules within the size range of 300-350 μ m were selected and used as the maximum size of microcapsules (Fig.6A).

DAPI staining

According to our data, the cells in groups A and D were stained the same at 24, 48, 72 hours and on day 7 with high viability. Accordingly, groups B and C did not differ significantly and showed less viability than that of groups A and D particularly after 24 hours. However, considerable differences were detected at 72 hours and on day 7 (Fig.4).

Releasing test

The reliable time was in day 7, on which the media was totally released (Fig.6B).

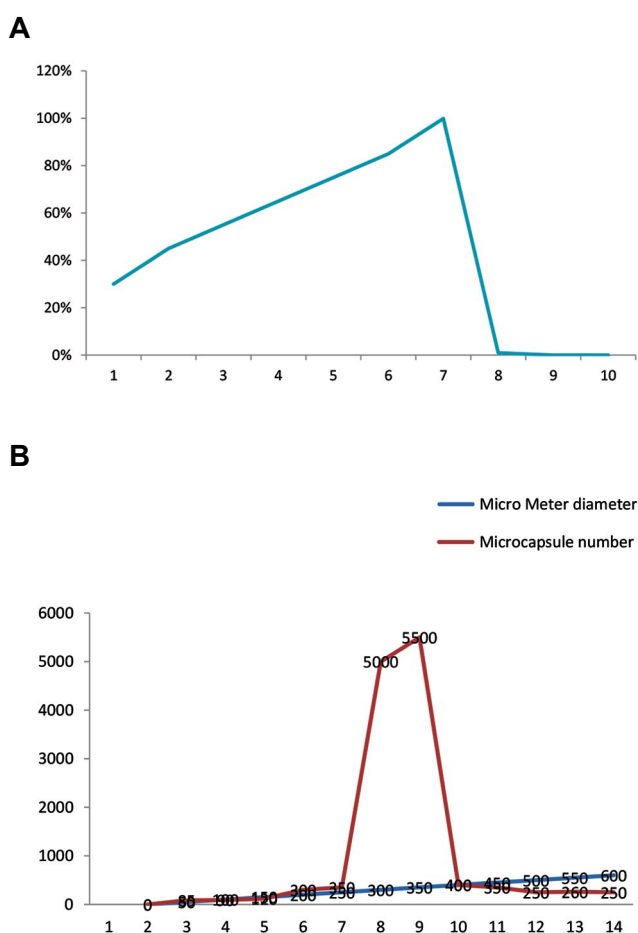


Fig.6: Diagrams of particle size and releasing time of microcapsules. **A.** Microcapsules particle size and **B.** Linear diagram of alginate microcapsules releasing time.

Discussion

In several studies, AM have been used as a supplier of nutrients for human fatty-derived mesenchymal stem cells. Alginates are polyanionic copolymers which have ionic interactions between the guluronic acid groups (6-9). The standard and effective substrate used for cell growth in cell culture is DMEM which is widely used for the growth of different mammalian cells (7). On the basis of different reports, many cells such as primary fibroblasts, neurons, glial cells, HUVECs and smooth muscle cells, as well

as cell lines like HeLa, 293, and Cos-7 were effectively cultured in DMEM (8).

DMEM was originally formulated with low glucose (1 g/L) and sodium pyruvate, but it is often used with higher glucose levels, with or without sodium pyruvate for cell differentiation. Gibco DMEM with GlutaMAX™ supplement minimizes toxic ammonia buildup and improves cell viability and growth (10-12). Sodium alginate is biologically safe and widely used in drug delivery systems (11). It is used to encapsulate various drugs in alginate beads (13, 14) and to delay matrix beads (15, 16). Controlling the release of dosage maintains a consistent therapeutic level of drug and minimizes the adverse effects. This is suitable for drug therapy as it can prolong the release of the drug over an extended period, reduce the frequency of administration, and increase patient compliance (17, 18).

Moshaverinia et al. (19) used the injectable oxidized alginate micro beads which encapsulated periodontal ligament (PDL) and gingival mesenchymal stem cells. As observed in the current study, AM were filled with DMEM using very fine needles. Then, they were added to mesenchymal stem cells. The null hypothesis was that MA might release nutritional media into cell environment and maintain the viability of stem cells for a short or long period. In the present research, MTT assay and DAPI staining were used to monitor adipose-derived mesenchymal stem cells (ADSCs) cell culturing. According to our data, similar results were detected in groups A and D. However, cells in groups B and C were visible after 24, 48, and 72 hours and after 7 days. The findings of the current study can be used as the basic information which can open a new window for alginate usage.

There is an increasing interest in microcapsules due to their wide applications in biomedicine (20), bioreactors, therapeutics, drug delivery system and tissue engineering (21). Among several materials available for production of microcapsules, the most commonly used microsphere is alginate (19, 22). Researchers have focused on stimuli-responsive polymeric-hydrogels using alginate due to its potential applications in drug delivery systems and tissue engineering (23-27). Lindenhayn et al. (28) used AM for cartilage tissue engineering and it was detected that AM protect stem cells. This effect is mediated by double-membrane microcapsules with a multi-enzyme system through self-assembly and bio-mineralized properties (26-29).

In the present study, AM (beads) were prepared as a reservoir containing nutrient media (DMEM) which was injected to AM. This method is noble and may be applicable in *in vitro* and *in vivo* studies or in clinical trials of cell therapy. The MTT and DAPI results showed that AM containing DMEM could support the cells for 7 days. The obtained results can be used as basic information and future studies should be conducted to find direct and cellular mechanism(s) underlying the protective

properties of AM on stem cells. Perhaps this technique can improve cell culture and cell therapy to be ultimately used in animal studies or clinical trials.

Conclusion

This achievement may open new horizons and create new approach for cell viability maintained on new condition of cell/stem cell culturing media (AM/media) with sustain release and good biocompatibility manner without refeeding consecutive. This may lead to protraction of cell viability on scaffold either in animals or humans body. The fruition of this survey can play vital role in the application of AM in tissue engineering and acts as a supplementary factor for nutrition 72 hours after implantation of scaffolds with cells in body. It can also be used as nutrient reservoir or nutrient pump or the carrier for cell differentiation cytokine in modern applied techniques. Briefly, nutrition media release of AM showed effective results. This study can create a new approach on the application of the AM in tissue engineering and cell transporting in animal or clinical trials.

Acknowledgments

Authors have to express their gratitude for the generous support of Dental School of Tehran University of Medical Sciences (TUMS) and Bioengineering Department of Nagasaki University. This study was financially supported by Research Deputy of TUMS under permission number 91-03-69-17701. The authors declare no conflict of interest.

Author's Contributions

A.Kh., E.D., F.N., A.V.; Synthesis and microcapsule loading-SEM-Particle size sorting. A.Kh., S.H.K., P.K.; Cell culture and Staining-Laboratory procedures. A.Kh., I.W., T.J.K.; Data analysis and writing. All authors read and approved the final manuscript.

References

- Wells LA, Sheardown H. Extended release of high pl proteins from alginate microspheres via a novel encapsulation technique. *Eur J Pharm Biopharm.* 2007; 65(3): 329-335.
- Maurstad G, Mørch YA, Bausch AR, Stokke BT. Polyelectrolyte layer interpenetration and swelling of alginate-chitosan multilayers studied by dual wavelength reflection interference contrast microscopy. *Carbohydr Polym.* 2008; 71(4): 672-681.
- Wee S, Gombotz WR. Protein release from alginate matrices. *Adv Drug Deliv Rev.* 1998; 31(3): 267-285.
- Smidsrod O, Skjak-Braek G. Alginate as immobilization matrix for cells. *Trends Biotechnol.* 1990; 8(3): 71-78.
- Bouhadir KH, Lee KY, Alsberg E, Damm KL, Anderson KW, Mooney DJ. Degradation of partially oxidized alginate and its potential application for tissue engineering. *Biotechnol Prog.* 2001; 17(5): 945-950.
- Moshaverinia A, Chen C, Xu X, Akiyama K, Ansari S, Zadeh HH, et al. Bone regeneration potential of stem cells derived from periodontal ligament or gingival tissue sources encapsulated in RGD-modified alginate scaffold. *Tissue Eng Part A.* 2014; 20(3-4): 611-621.
- Balakrishnan B, Mohanty M, Umashankar PR, Jayakrishnan A. Evaluation of an in situ forming hydrogel wound dressing based on oxidized alginate and gelatin. *Biomaterials.* 2005; 26(32): 6335-4632.
- Xin L, Li-ren C. Study on the permeability and swellability of alginate-chitosan microcapsules. *Journal of Yunnan University.* 2011; 33(3): 325-327.
- Wong DW S, Gregorski KS, Hudson GS, Pavlath AE. Calcium alginate films: thermal properties and permeability to sorbate and ascorbate. *Journal of Food Science.* 1996; 61(2): 337-341.
- Debnath T, Shalini U, Kona LK, Vidya Sagar JVS, Kamaraju SR, Gaddam S, et al. Development of 3D alginate encapsulation for better chondrogenic differentiation potential than the 2D pellet system. *J Stem Cell Res Ther.* 2015; 5(4): 276.
- Fundueanu G, Nastruzzi C, Carpov A, Desbrieres J, Rinaudo M. Physico-chemical characterization of Ca-alginate microparticles produced with different methods. *Biomaterials.* 1999; 20(15): 1427-1435.
- Serp D, Cantana E, Heinzen C, Von Stockar U, Marison IW. Characterization of an encapsulation device for the production of mono-disperse alginate beads for cell immobilization. *Biotechnol Bioeng.* 2000; 70(1): 41-53.
- Thu B, Bruheim P, Espevik T, Smidsrod O, Soon-Shiong P, Skjak-Braek G. Alginate polycation microcapsules. I. Interaction between alginate and polycation. *Biomaterials.* 1996; 17(10): 1031-1040.
- Sriamornsak P, Thirawong N, Korkeerd K. Swelling, erosion and release behavior of alginate-based matrix tablets. *Eur J Pharm Biopharm.* 2007; 66(3): 435-450.
- Cui JH, Goh JS, Park SY, Kim PH, Le BJ. Preparation and physical characterization of alginate microparticles using air atomization method. *Drug Dev Ind Pharm.* 2001; 27(4): 309-319.
- Halder A, Maiti S, Sa B. Entrapment efficiency and release characteristics of polyethyleneimine-treated or -untreated calcium alginate beads loaded with propranolol-resin complex. *Int J Pharm.* 2005; 302(1-2): 84-94.
- Nokhodchi A, Taylor A. In situ cross-linking of sodium alginate with calcium and aluminum ions to sustain the release of theophylline from polymeric matrices. *Farmaco.* 2004; 59(12): 999-1004.
- Mandal S, Basu SK, Sa B. Sustained release of a water-soluble drug from alginate matrix tablets prepared by wet granulation method. *AAPS Pharm Sci Tech.* 2009; 10(4): 1348-1356.
- Moshaverinia A, Chen C, Akiyama K, Ansari S, Xu X, Chee WW, et al. Alginate hydrogel as a promising scaffold for dental-derived stem cells: an in vitro study. *J Mater Sci Mater Med.* 2012; 23(12): 3041-3051.
- Wellingand PG, Dobrinska MR. Dosing considerations and bio-availability assessment of controlled drug delivery systems. In: Robinson GR, Lee VHL, editors. *Controlled drug delivery.* 2nd ed. USA: CRC Press; 1987; 253-291.
- Behme RJ, Brooke D, Farney RF, Kensler TT. Characterization of polymorphism of gepirone hydrochloride. *J Pharm Sci.* 1985; 74(10): 1041-1046.
- Costa RR, Mano JF. Polyelectrolyte multilayered assemblies in biomedical technologies. *Chem Soc Rev.* 2014; 43(10): 3453-4379.
- Mak WC, Bai J, Chang XY, Trau D. Matrix-assisted colloidosome reverse-phase layer-by-layer encapsulating biomolecules in hydrogel microcapsules with extremely high efficiency and retention stability. *Langmuir.* 2009; 25(2): 769-775.
- De Temmerman ML, Demeester J, De Vos F, De Smedt SC. Encapsulation performance of layer-by-layer microcapsules for proteins. *Biomacromolecules.* 2011; 12(4): 1283-1289.
- Abidian MR, Corey JM, Kipke DR, Martin DC. Conducting-polymer nanotubes improve electrical properties, mechanical adhesion, neural attachment, and neurite outgrowth of neural electrodes. *Small.* 2010; 6(3): 421-429.
- Abidian MR, Kim DH, Martin DC. Conducting-polymer nanotubes for controlled drug release. *Adv Mater.* 2006; 18(4): 405-409.
- Shi J, Zhang L, Jiang Z. Facile construction of multicompartment multienzyme system through layer-by-layer self-assembly and biomimetic mineralization. *ACS Appl Mater Interfaces.* 2011; 3(3): 881-889.
- Lindenhayn K, Perka C, Spitzer R, Heilmann H, Pommerening K, Mennicke J, et al. Retention of hyaluronic acid in alginate beads: aspects for in vitro cartilage engineering. *J Biomed Mater Res.* 1999; 44(2): 149-155.
- Keshel SH, Soleimani M, Tavirani MR, Ebrahimi M, Raeisossadati R, Yasaei H, et al. Evaluation of unrestricted somatic stem cells as a feeder layer to support undifferentiated embryonic stem cells. *Mol Reprod Dev.* 2012; 79(10): 709-718.