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Comparative Epigenetic Analysis of Imprinting Genes Involved in Fertility, in Cryopreserved Human Sperms with Rapid Freezing versus Vitrification Methods

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Abstract

Objective: Choosing the optimal method for human sperm cryopreservation seems necessary to reduce cryoinjury. The aim of this study is to compare two cryopreservation methods including rapid-freezing and vitrification, in terms of cellular parameters, epigenetic patterns and expression of paternally imprinted genes (*PAX8, PEG3* and *RTL1*) in human sperm which play a role in male fertility.

Materials and Methods: In this experimental study, semen samples were collected from 20 normozoospermic men. After washing the sperms, cellular parameters were investigated. DNA methylation and expression of genes were investigated using methylation-specific polymerase chain reaction (PCR) and real-time PCR methods, respectively.

Results: The results showed a significant decrease in sperm motility and viability, while a significant increase was observed in DNA fragmentation index of cryopreserved groups in comparison with the fresh group. Moreover, a significant reduction in sperm total motility (TM, P<0.01) and viability (P<0.01) was determined, whereas a significant increase was observed in DNA fragmentation index (P<0.05) of the vitrification group compared to the rapid-freezing group. Our results also showed a significant decrease in expression of *PAX8, PEG3* and *RTL1* genes in the cryopreserved groups compared to the fresh group. However, expression of PEG3 (P<0.01) and *RTL1* (P<0.05) genes were reduced in the vitrification compared to the rapid-freezing group. Moreover, a significant increase in the percentage of *PAX8, PEG3* and *RTL1* methylation was detected in the rapid-freezing group (P<0.01, P<0.0001 and P<0.001, respectively) and vitrification group (P<0.01, P<0.0001 and P<0.0001, respectively) compared to the fresh group. Additionally, percentage of *PEG3* and *RTL1* methylation in the vitrification group was significantly increased (P<0.05 and P<0.05, respectively) compared to the rapid-freezing group.

Conclusion: Our findings showed that rapid-freezing is more suitable method for maintaining sperm cell quality. In addition, due to the role of these genes in fertility, changes in their expression and epigenetic modification may affect fertility.

Keywords: Epigenetics, Male Fertility, Rapid-Freezing, Vitrification

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Introduction

Male infertility is a complex disease, resulted from interaction between genetic and environmental factors. Several male factors, such as spermatogenesis, sperm function, sperm immotile, sperm with abnormal morphology, sexual activity and endocrine system can affect male infertility. Although 30% of the caused infertility are related to male factors, about 10% of the male infertility are diagnosed with unexplained or idiopathic infertility (1). Genetic mutations in sperm and changes in epigenetic patterns are important factors in male infertility (2). Thus, assisted reproductive techniques (ART), including sperm cryopreservation, are useful to preserve male fertility and treat infertility in men (3). However, studies have shown that cellular and molecular changes occur during cryopreservation (4). Different methods, such as rapid-freezing (liquid nitrogen vapor cooling followed by plunging into liquid nitrogen)

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and vitrification (directly plunging into liquid nitrogen) have been suggested to perform sperm cryopreservation (5). Isachenko et al. (6) reported that vitrification was extremely fast and there was not enough time for formation of ice crystals. Extremely high rate of temperature reduction prevented formation of intracellular ice crystals, but more studies are needed to demonstrate efficiency of this method. Despite the research efforts undertaken to develop improved human semen cryopreservation methods, it is yet unknown whether one technique is advantageous over the other (3). So far, several studies have compared these methods based on the sperm quality parameters after thawing. In addition, previous studies on the sperm of various animals showed that some epigenetic patterns, including DNA methylation and expression of some sperm genes, were changed after cryopreservation (7-9). But, studies performed to determine changes in gene expression and epigenetic patterns during different cryopreservation methods are limited (10).

Many imprinted genes, such as *PAX8*, *PEG3* and *RTL1*, are involved in sperm function and fertility11-) 13). Since defective methylation of sperm DNA and changes in the epigenetic pattern can be the main reasons underlying decline in fertility potential of sperm after thawing (14), in this study we aimed to compare effect of two cryopreservation protocols, rapid-freezing and vitrification on cell viability, mobility, morphology, DNA fragmentation, DNA methylation status and expression of *PAX8*, *PEG3* and *RTL1* genes in human sperm.

Materials and Methods

Sample collection and preparation

Semen samples were collected from 20 healthy men (normozoospermia) with 20-35 years of age, who referred to Royan Infertility Clinic in Tehran, Iran. All patients were informed about the methods of study and agreed analysis of genetic material for research purposes, while informed written consent was obtained from all patients. This study was approved by the Ethics Committee of Royan Institute (IR.ACECR.ROYAN.REC.1400.147). The exclusion criteria for this study were presence of varicocele, cigarettes smoking, alcohol consumption and exposure to chemotherapy or radiation as well as specific diseases including diabetes. Semen samples were collected in sterile semen collection tubes after 3-4 days' abstinence. After liquefaction of the samples at 37°C for 30 minutes, a basic semen analysis was undertaken within one hour according to the 2010 World Health Organization (WHO) guidelines (15). The criteria for identification as normal quality sperm were consisted of morphology (≥4% normal), concentration ($\geq 15\times10^6$ sperm/ml), motility (≥40% motile), progressive motility (PM, ≥32%) and normal viscosity (15). Recently, in most fertility centers, semen preparation is performed by swim-up or DGC method to obtain high quality sperm. For this purpose, all investigations were carried out on spermatozoa, prepared by the density gradient centrifugation technique (DGC) (16). Semen was layered on 80% Allgrade (Life Global, USA) and 20% human tubal fluid. After centrifugation at 1800 rpm for 15 minutes, the supernatant was discarded and sperms were collected from the pellet. After DGC technique, the collected pellet was resuspended in the basic medium to achieve a concentration of 60×10^6 spermatozoa/ml and finally aliquoted into three equal sub-samples for three different groups (fresh as a control group, frozen groups including rapid-freezing and vitrification). After cryopreservation, normal cells, including viable, motile sperm cells, with normal morphology, were used for analyses.

Cryopreservation

Methods for cryopreservation were according to the previous studies (17, 18). In the frozen group by rapid-freezing method, SpermFreeze™ Solution (FertiPro N.V., Belgium; contains physiologic salts, glycine, glucose, lactate, 15% glycerol, sucrose and 4.0 g/l human serum albumin) was added in droplets to the washed sperm suspension at a ratio of 0.7:1 v/v. The samples were then kept at room temperature for 10 minutes. The mixture was transferred to a volume of 1.0 ml of cryotube (Nunc, Denmark). The cryotubes were suspended in a liquid nitrogen vapor phase (15 cm above the liquid nitrogen) for 15 minutes. Then, cryotubes were immersed into a liquid nitrogen tank for storage and they were stored for 1 week (18).

In vitrification method, SpermFreeze solution was added in droplets to the washed sperm suspensions at a ratio of 0.7:1 v/v and they were kept at room temperature for 10 minutes. They were subsequently loaded into a straw (IMV, France) and immersed directly into a liquid nitrogen tank and stored for one week (17).

The samples were thawed in a water bath at 37˚C for 5 minutes, furthermore, each sample was centrifuged at 3500 rpm for 5 minutes, the upper portion containing freezing medium was removed and 1ml of human tubal fluid medium (Sigma, USA) supplemented with 5% human serum albumin (Sigma, USA) were added to the sperm pellets and incubated at 37°C for 20 minutes before sperm quality parameter analysis (18).

Sperm motility analysis

Sperm motility parameters were measured using the Semen Class Analysis Software (SCA, version 5.1; Microptic, Spain) (19). A total of 5 µl of the sperm sample was placed on a pre-warmed chamber slide (37°C) under a microscope; they were analyzed for sperm motion characteristics including total motility (TM, %), progressive motility (PM, %), curvilinear velocity (VCL, µm/s), average path velocity (VAP, µm/s), amplitude of straightness (STR, %), velocity straight linear (VSL, µm/s) and linearity (LIN, %).

Morphology assessment

Papanicolaou staining was used for assessment of sperm morphology. Briefly, 20 µl of sperm suspension smear

was made on a glass slide and dried at room temperature. The samples were then fixed in methyl alcohol (70%) and immersed in hematoxylin solution for 3 minutes. They were next rinsed in water and immersed in alcohol (95%). Subsequently, they were immersed in OG-6 stain for 1.5 minutes, ethanol (95%) 10 dips and EA-50 stain for 2.5 minutes. After washing in water, they were immersed in alcohol (96%) for three times. Upon drying at room temperature, each slide was assessed under oil immersion light microscopy at 400x magnification, by counting at least 200 sperm. Mean percentage of normal morphology of spermatozoa was determined (20).

Sperm viability

To evaluate viability, an equal amount of eosin-negrosin stain and sperm suspension were poured into a 0.5 ml microtube and pipetted several times to mixed together. After 4 minutes, a smear was made using the drops of 5 µl solution on a warm slide and they were dried at room temperature. Sperm viability was assessed by counting 200 spermatozoa on each slide under oil immersion light microscopy at 400x magnification. The sperm cells that showed regional or complete purple staining were considered as nonviable and mean percentage of spermatozoa with membrane intactness (unstained) was defined as live spermatozoa (14).

DNA fragmentation

Sperm DNA fragmentation index was measured using the sperm DNA fragmentation assay kit (SDFA; ACECR, Iran) protocol. Thirty µl of the sperm suspension was dissolved in the melted agarose and one drop (50 µl) was placed on a slide. Then, the sample was immersed in HCl solution.

After washing with water and ethanol (70, 90 and 100%), the sample was immersed in lysing solution. A total of 200 spermatozoa per sample were evaluated under a light microscope (Olympus CX21; Olympus, USA) at a magnification of 1000x (21).

RNA extraction and quantitative reverse transcription polymerase chain reaction

Total RNA was extracted from the sperm pellets using the RNA Micro Kit (Cat. No. 74004; Qiagen, Germany) as described by the manufacturer's protocol. In order to isolate total RNA from the sperms, we used TRIzol reagent and to separate the proteins, three steps of phenol-chloroform purification were done. In order to purify and evaluate RNA concentration, we used NanoDrop ND-1000® spectrophotometry (Thermo Fisher Scientific Inc., USA). Excel RT Reverse Transcription Kit-Rp1300 (Zist Fanavari Pishgam, Iran) kit was used to synthesize the first strand of cDNA by adding 100 ng/µl of the purified total RNA, while it was carried out in a total volume of 20 µl containing, 1 µl dNTP, 1 µl Random hexamer, 8 µl Purified total RNA, 4 µl RT buffer (1 X), 4 µl DEPC, 1 µl RNase inhibitor and 1 µl RT enzyme. Primer sequences were designed using PerlPrimer (Version: 1.1.21; SourceForge, USA) and GeneRunner (Version: 6.5.52; GeneRunner.net) software for:

PAX8-
F: 5′-GTCCTCTTACTCTAAGCCCA-3′
R: 3′-CCACACTACACTCTACCTCTC-5′

PEG3-
F: 5′-ACACATATTCCCAACACCCA-3′
R: 3′-CCATAATCCCACAACAACCAC-5′

RTL1-
F: 5′-CAACAGACAGGACTACATACAG-3′
R: 3′-CATCTCTTCAAGCTCCAAACC-5′.

Then, quantitative reverse transcription PCR (qRT-PCR) was carried out for two technical repeats on StepOne™ Real-Time PCR System (Applied Biosystems, USA) in a total volume of 10 µl containing, 3 µl cDNA, 1 µl of each primer and 2.5 µl SYBR Green master mix (Ampliqon real Q plus 2x; Ampliqon, Korea). Reaction was performed under the following program: initial denaturation at 95˚C for 5 minutes, followed by 45 cycles of denaturation at 95˚C for 15 secends, annealing at 60˚C for 25 seconds and extension at 72˚C for 25 seconds. Then, expression levels of the *PAX8*, *PEG3* and *RTL1* genes was evaluated using Ct values. Relative gene expression was assessed using *β-Actin* and $2^{-\Delta\Delta Ct}$ method (22).

DNA extraction and bisulfite treatment

Total sperm DNA was extracted of the sperm pellets using the DNA extraction Kit (DNrich Sperm 1012; Azma Elixir Pajooh, Iran) according to the kit instructions. Quantity of the extracted DNA was evaluated by the NanoDrop ND-1000® spectrophotometry. Then, 1-2 µg of the extracted total DNA was treated with sodium bisulfite method, as previously described by Yi et al. (23) with some modifications (24). Bisulfite treated DNA solution was purified with the FavorPrep GEL/PCR Purification Mini Kit (Cat. No: FAGCK001-2; Favorgen, Taiwan). The bisulfite-treated DNA was then analyzed using methylation-specific PCR (25, 26).

Methylation analysis using methylation-specific polymerase chain reaction assay

Difference in methylation status was measured by methylation-specific PCR (MSP) method (25, 26). Primer sequences used in the MSP method (methylated and unmethylated sequences) were designed using MethPrimer2 software (MethPrimer2.0; urogene, China) for:

PAX8-
F: 5′-TTTCGTTTAGTTTATGGAGAGGC-3′
R: 3′-GAAACGTCGTCGTACAACGT-5′
for methylated sequence,

F: 5′-TGTTTAGTTTATGGAGAGGTGG-3′
R: 3′-TAACAAAACATCATCATACAACATC-5′
for unmethylated sequence,

PEG3-

F: 5′-GGTAATCGTAGTTTGATTGGTACGT-3′
R: 3′-AAACTTCTCCGCAAAAACGA-5′
for methylated sequence,

F: 5′-GTGGTAATTGTAGTTTGATTGGTATGT-3′
R: 3′-TTATCAAAACTTCTCCACAAAAACA-5′
for unmethylated sequence,

RTL1-
F: 5′-TTTTTATTTTGGAAAGTCGGTTATC-3′
R: 3′-AACTACGACAAATACGTACGATACG-5′
for methylated sequence,

F: 5′-TTTATTTTGGAAAGTTGGTTATTGG-3′
R: 3′-CACTTTTAACTACAACAAATACATACAAT-5′
for unmethylated sequence.

Briefly, in a final volume of 25 µl reaction, 2 µl DNA was amplified with primers specific to methylated and unmethylated sequences. Then, the following cycling PCR conditions were applied: pre- denaturation at 94°C for 5 minutes, 35 cycles of denaturation at 94°C for 30 seconds, primer annealing at 60°C for 30 seconds and primer extension at 72°C for 1 minute. Final extension was performed in 4°C for 7 minutes, 30 cycles. In all experiments, fully methylated DNA and fully unmethylated DNA were employed as positive controls and DNase/RNase-free distilled water was used for the negative PCR control. Then, a volume of 10 µl of each PCR products was visualized on 2% agarose gel at Voltage 95 V for 70 minutes, with general 1X TBE buffer and DNA bands were visualized by UV-transilluminators. Band intensities of each sample was analyzed using ImageJ (NIH corporation, USA) and GraphPad Prism 8 (GraphPad PRISM V 8 analytical software; GraphPad corporation, USA) software (27).

Statistical analysis

Statistical analysis was performed using SPSS software (version 26 for Windows; IBM-SPSS Statistics, IBM Corporation, USA) and GraphPad Prism 8 software (GraphPad corporation, USA). Normality analysis of the data was evaluated with the Kolmogorov–Smirnov test. Data were analysis using one-way ANOVA. Post hoc comparisons were assessed by Kruskal-Wallis (non-parametric) and Tukey (parametric) tests. Statistically significant was considered as $P<0.05$.

Results

Effects of rapid-freezing and Vitrification methods on frozen–thawed sperm motility parameters

As shown in Table 1, percent of TM, PM, VAP, VCL and straight-line velocity (VSL) were significantly decreased in the rapid-freezing (42.97 ± 2.3, 29.03 ± 1.2, 31.34 ± 0.6, 51.69 ± 0.8 and 26.66 ± 0.5, respectively) and vitrification groups (26.35 ± 1.5, 18.43 ± 0.5, 28.74 ± 1.3, 46.15 ± 1.4 and 22.98 ± 1.2, respectively) compared to the fresh group.

Additionally, according to Table 1 and the mentioned results above, motility parameters in the vitrification group was significantly decreased compared to the rapid-freezing group (TM: $P<0.01$, PM: $P<0.01$, VCL: $P< 0.05$ and VSL: $P<0.05$).

Effects of rapid-freezing and vitrification methods on viability, morphology and DNA fragmentation

Effect of different cryopreservation methods on viability, morphology and DNA fragmentation are presented in Figure 1. Comparison of the mean values for sperm viability in the rapid-freezing group (61.95 ± 0.8) and vitrification group (42.15 ± 1.9) showed a significant reduction ($P<0.001$ and $P<0.0001$, respectively) compared to the fresh group (90.10 ± 0.6). In addition, results showed a significant reduction ($P<0.01$) in sperm viability of the vitrification group compared to the rapid-freezing group (Fig.1A). Normal morphology was not significantly affected by different frozen methods ($P>0.05$, Fig.1B).

DNA fragmentation index showed a significantly increase in the rapid-freezing (25.45 ± 1.3, $P<0.0001$) and vitrification groups (29.80 ± 1.2, $P<0.0001$) compared to the fresh group (8 ± 0.6). Moreover, the vitrification group showed a significantly increase ($P<0.05$) compared to the rapid-freezing group (Fig.1C).

Table 1: Effect of different cryopreservation (rapid and vitrification) methods on frozen-thawed human spermatozoa motility parameters

Motility parameters Groups	Total motility (%)	PM (%)	VAP (µm/s)	VCL (µm/s)	VSL (µm/s)	LIN (%)	STR (%)
Fresh semen	81.66 ± 1.584	66.54 ± 1.553	43.07 ± 0.8208	73.16 ± 1.711	36.22 ± 0.7510	51.98 ± 1.568	84.13 ± 0.8660
Rapid-freezing	42.97 ± 2.317***	29.03 ± 1.272***	31.34 ± 0.6765****	51.69 ± 0.8928****	26.66 ± 0.5509****	53.68 ± 1.000	85.32 ± 1.464
Vitrification	26.35 ± 1.540****	18.43 ± 0.5917****	28.74 ± 1.322****	46.15 ± 1.477****	22.98 ± 1.282****	51.66 ± 2.283	81.58 ± 3.435

Data are presented as mean ± SE. PM; Progressive motility, VAP; Average path velocity, VCL; Curvilinear velocity, VSL; Straight linear velocity, LIN; Linearity, STR; Amplitude of straightness, ***; P<0.001, and ****; P<0.0001.

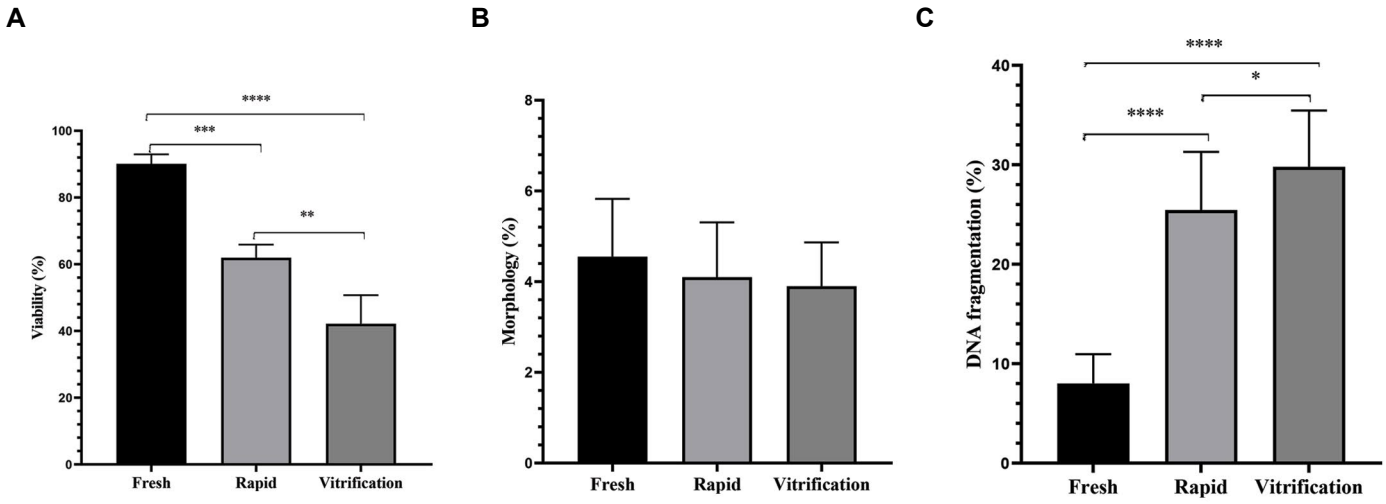

Fig.1: Effects of different cryopreservation groups on sperm cell parameters. **A.** Viability, **B.** Morphology and **C.** DNA fragmentation. *; P<0.05, **; P<0.01, ***; P<0.001, and ****; P<0.0001.

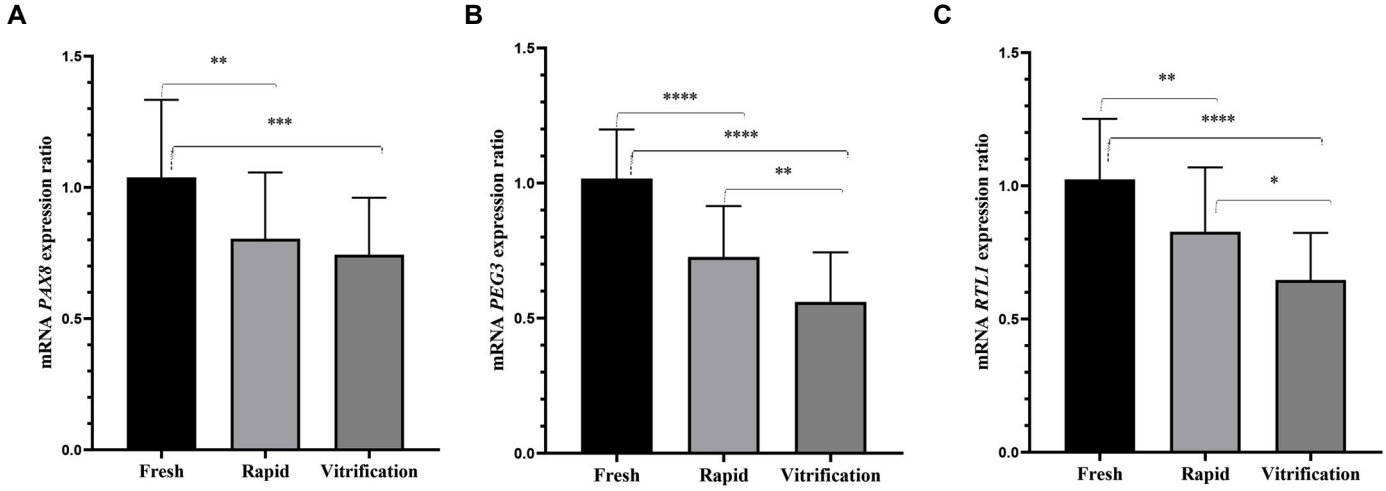

Fig.2: Assessment of gene expression in the cryopreservation groups. We used quantitative reverse transcription polymerase chain reaction (qRT-PCR) experiments to measure expression of the indicated genes. mRNA expression levels of **A.** *PAX8*, **B.** *PEG3*, **C.** *RTL1*. *; P<0.05, **; P<0.01, ***; P<0.001, and ****; P<0.0001.

Effects of the rapid-freezing and vitrification methods on expression of *PAX8*, *PEG3*, and *RTL1* genes

Our results (Fig.2A) showed that expression level of *PAX8* gene in the rapid-freezing (0.80 ± 0.05) and vitrification (0.74 ± 0.04) groups were significantly reduced (P=0.006 and P=0.0005, respectively) compared to the fresh group (1.03 ± 0.06). *PAX8* expression level was not significantly different between the two cryopreservation methods (P=0.694).

Expression level of *PEG3* gene was decreased significantly (P<0.0001) in the rapid-freezing (0.72 ± 0.03) and vitrification (0.56 ± 0.03) groups compared to the fresh group (1.01 ± 0.03). Furthermore, in the vitrification group (0.56 ± 0.03) expression level of *PEG3* gene was decreased significantly (P=0.0074) compared to the rapid-freezing group (0.72 ± 0.03). Assessment of *PEG3* gene expression is presented in Figure 2B.

According to the results in Figure 2C, it was found that expression level of *RTL1* gene in the rapid-freezing group (0.82 ± 0.04) and vitrification groups (0.64 ± 0.03) were decreased significantly (P=0.007 and P<0.0001, respectively) compared to the fresh group (1.02 ± 0.04). Additionally, expression level of *RTL1* gene was significantly reduced in the vitrification group (0.64 ± 0.03) compared to the rapid group (0.82 ± 0.04, P=0.014).

Effects of rapid-freezing and vitrification methods on *PAX8*, *PEG3* and *RTL1* gene promoter methylation status

To analyze gene promoter methylation status, PCR products were loaded on 2% agarose gels (Fig.3A, 4A, 5A) and after analyzing methylation data, ratio between methylated and unmethylated band intensities was calculated (Fig. 3B, 4B, 5B).

According to the results (Fig.3), it was found that percentage of *PAX8* methylation in the rapid-freezing (9.96 ± 0.4) and vitrification groups (9.94 ± 0.4) were significantly increased (P=0.0015 and P=0.0014, respectively) compared to the fresh group (7.34 ± 0.4).

Results of *PEG3* gene promoter methylation status in the cryopreservation and fresh groups are shown in Figure 4. As shown in Figure 4, percentage of *PEG3* gene methylation in the rapid-freezing (12.80 ± 0.7) and vitrification groups (15.17 ± 0.7) were increased significantly (P<0.0001 and P<0.0001, respectively) compared to the fresh group (5.66 ± 0.4). The results also showed that percentage of *PEG3* methylation in the vitrification group (15.17 ± 0.7) was significantly increased compared to the rapid-freezing group (12.80 ± 0.7, P=0.478).

As shown in Figure 5, percentage of *RTL1* methylation in the rapid-freezing (11.37 ± 0.7) and vitrification groups (14.10 ± 0.8) were increased significantly (P=0.0002 and P<0.0001, respectively) compared to the fresh group (7.320 ± 0.3). Besides, in the vitrification group (14.10 ± 0.8), percentage of *RTL1* methylation was significantly increased (P=0.0165) compared to the rapid-freezing group (11.37 ± 0.7).

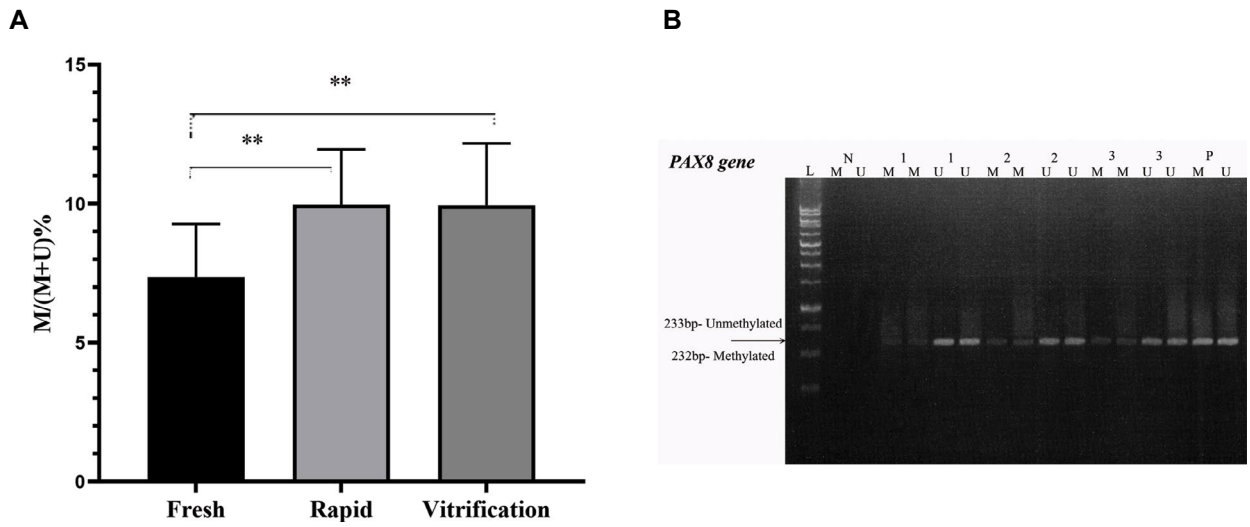

Fig.3: Methylation-specific PCR (MSP) analysis of *PAX8* gene. **A.** Percentage of *PAX8* gene promoter methylation in sperm cells after freeze-thawing process. Fresh; Control group including fresh sperm samples, Rapid; Rapid-freezing group, Vitrification; Vitrification group, and **; P<0.01. **B.** PCR products loaded on 2% agarose gel. PCR; Polymerase chain reaction, N; Negative PCR control including distilled water (for unmethylation and methylation PCR, P; Positive control including complete methylated DNA (for methylation and unmethylation PCR), M; Methylated amplicon, U; Unmethylated amplicon, Samples 1; Fresh groups, Samples 2; Rapid groups, and Samples 3; Vitrification groups.

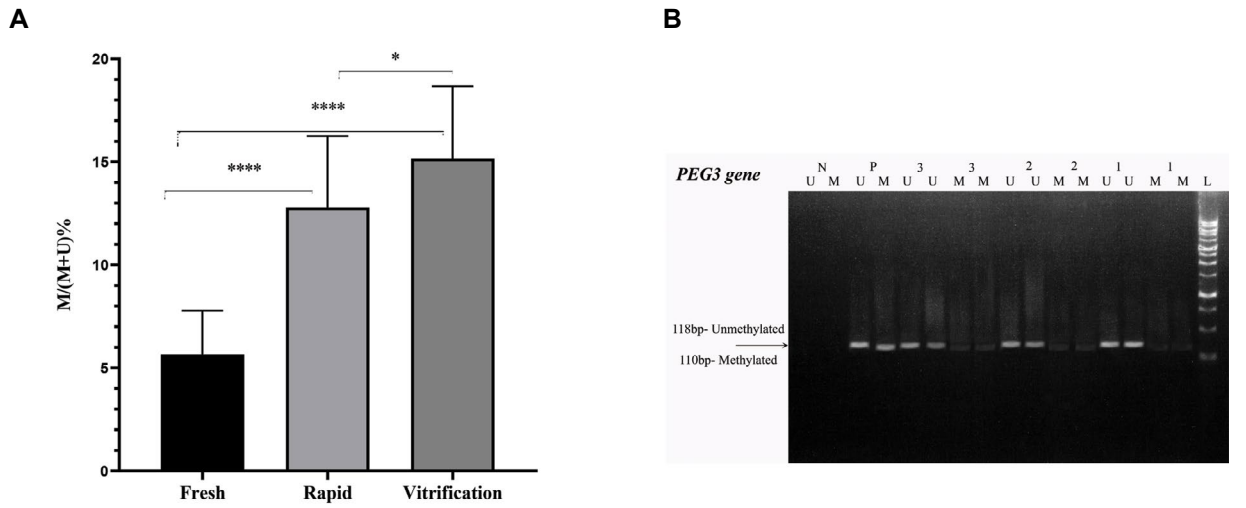

Fig.4: Methylation-specific PCR (MSP) analysis of *PEG3* gene. **A.** Percentage of *PEG3* gene promoter methylation in sperm cells after freeze-thawing process. Fresh; Control group including fresh sperm samples, Rapid; Rapid-freezing group, Vitrification; Vitrification group, *; P<0.05, ****; P<0.0001. **B.** PCR products loaded on 2% agarose gel. PCR; Polymerase chain reaction, N; Negative PCR control including distilled water (for unmethylation and methylation PCR), P; Positive control including complete methylated DNA (for methylation and unmethylation PCR), M; Methylated amplicon, U; Unmethylated amplicon, Samples 1; Fresh groups, Samples 2; Rapid groups, and Samples 3; Vitrification groups.

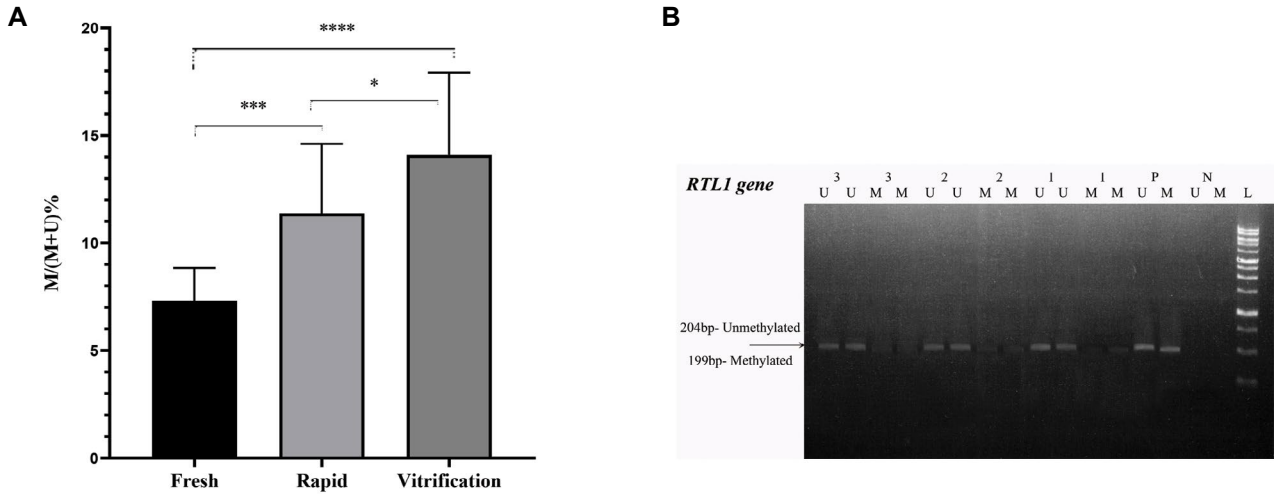

Fig.5: Methylation-specific PCR (MSP) analysis of *RTL1* gene. **A.** Percentage of *RTL1* gene promoter methylation in sperm cells after freeze-thawing process. Fresh; Control group including fresh sperm samples, Rapid; Rapid-freezing group, Vitrification; Vitrification group, *; P<0.05, ***; P<0.001, and ****, P<0.0001. **B.** PCR products loaded on 2% agarose gel. PCR; Polymerase chain reaction, N; Negative PCR control including distilled water (for unmethylation and methylation PCR), P; Positive control including complete methylated DNA (for methylation and unmethylation PCR), M; Methylated amplicon, U; Unmethylated amplicon, Samples 1; Fresh groups, Samples 2; Rapid groups, and Samples 3; Vitrification groups.

Discussion

In the recent years, sperm storage are important in the management and treatment of male infertility, but studies show that sperm quality and fertility potential are greatly reduced after freeze-thawing process. According to the previous studies, sperm quality parameters and macromolecular agents are impaired after freezing-thawing (14). Some environmental and epigenetic factors, such as temperature changes, environmental pollutants and chemicals have an effect in male infertility. So, genetic- (such as mutation-) and epigenetic-based disorders affecting spermatogenesis may be responsible for most of the idiopathic infertility cases (28). Therefore, it is necessary to determine a suitable freezing method which can reduce cryoinjury in thawed sperm. In the present study, a significant increase was observed in the DNA fragmentation index after freeze-thawing compared to the fresh group. Our findings also showed a significant increase in DNA fragmentation index of the vitrification compared to the rapid-freezing group. The main causes of sperm DNA fragmentation are increased levels of oxidative stress, protamine deficiency during chromatin packaging and apoptosis which can be related to the infertility (29). Mechanisms explaining reason of the increase in DNA fragmentation after freezing have not been identified yet, but it seems to be associated with cold shock that increases oxidative DNA damage (30). Our results showed a significant reduction in motility parameters of thawed sperm. Our findings support results of the previous studies reported that sperm motility and viability were decreased after freeze-thawing process (30-32). It has been reported that destructive effect on the mitochondria and plasma membrane can reduce sperm motility and viability after thawing (30). Furthermore, our results showed a significant decrease of sperm motility and viability in the vitrification compared to the rapid-freezing group. Our findings are in line with the results of previous studies, reported that sperm freezing by the vitrification method could reduce sperm motility and survival compared to the rapid-freezing method (33, 34). Le et al. (34) reported that PM and vitality were significantly higher in the rapid-freezing than vitrification method. Agha-Rahimi et al. (33) also compared effects of the rapid-freezing and vitrification methods on various sperm parameters. They reported that the vitrification method was not superior to the rapid-freezing of the normozoospermic spermatozoa. These results could be related to the vitrification procedure and quality of sperm that are important factors to increase the effectiveness of it (35).

In addition to damaging cellular parameters, cryopreservation can affect sperm macromolecules, such as proteins, transcripts and epigenetic patterns. Epigenetic modification, especially methylation, can cause changes in gene expression and gene silencing. In our research, a significant reduction was observed in expression of the three (*PAX8*, *PEG3* and *RTL1*) genes during the rapid-freezing and vitrification methods. Although, the highest reduction was related to the vitrification group. Our gene expression results corresponded to the previous studies, reported that expression of some sperm genes were changed after freezing compared to the fresh sperm (7, 10).

Changes in temperature, such as freezing could affect DNA methylation (36). Previous studies on the sperm of various animals, after the cryopreservation process showed epigenetic modification, including DNA methylation, after freezing (8, 9, 22) which is consistent with our results. In this study, we found that percentage of *PAX8*, *PEG3* and *RTL1* methylation was increased significantly in the rapid-freezing and vitrification groups compared to the fresh group. These results also showed that percentage of *PEG3* and *RTL1* methylation in the vitrification group was increased compared to the rapid-freezing group. Our findings supported results

of the previous studies reported that mouse embryos obtained from the cryopreserved sperm had higher DNA methylation rates than the embryos obtained from fresh sperm (22). Aurich et al. (8) also reported that sperm DNA methylation was increased in horses after freezing, which affected their successful fertility. It has been reported that cryopreservation caused epigenetic changes in the boar sperm (9). As mentioned before, *PAX8, PEG3* and *RTL1* genes have roles in male fertility and epigenetic modifications of mature sperm played an important role in the embryonic development (11-13). Previous studies reported that hypermethylation of *PEG3* gene was associated with infertility in oligospermic men (37), while changes in the level of methylation of this gene was observed in sterile male (11). Furthermore, *PEG3* gene played an important role in controlling fetal growth (38). In line with this, our results showed a decrease in the *PEG3* gene expression and hypermethylation in this gene, which may affect embryonic development. On the other hand, studies showed that hypermethylation of *PAX8* gene reduced sperm concentration and motility and it caused abnormal sperm morphology (12). Decreased expression of this gene was observed in the infertile mice (39). Due to the role of this gene in regulating synthesis of thyroid hormones (40), decreased expression and hypermethylation of this gene maybe impaired sperm production. Previous researchers reported that *RTL1* gene had a different methylation pattern in abnormal sperm compared to the fertile men (13). Our findings showed decreased expression and hypermethylation in this gene, which may lead to the impaired successful fertility. The main limitation in this project was lack of studies and resources to compare results of different freezing methods on methylation of paternal imprinting genes.

Conclusion

Our findings showed that rapid-freezing is a more suitable method for maintaining sperm cell quality after freeze-thawing process, compared to the vitrification method. Since epigenetic modifications of the imprinted genes is the main reason underlying decline in sperm motility and fertility during the freeze-thawing process, our findings indicated that rapid-freezing was a better method for sperm protection against cryo-damages compared to the vitrification method. Due to the role of *PAX8*, *PEG3* and *RTL1* genes in fertility and embryonic development, changes in the expression and epigenetic modification of these genes may affect success of fertility and embryo development, which probably can be one of the reasons for male infertility.

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Authors' Contributions

M.Sh., V.H.; Supervision. N.Kh., V.H., M.Sh., M.H., A.Sh.; Conceptualization, Methodology, Data curation, Validation, Writing-reviewing and Editing resources. N.Kh.; Software, Formal analysis, and Writing-original draft preparation. N.Kh., M.H.; Investigation and Visualization. All authors read and approved the final manuscript.

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