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Effects of Vitrification on Nuclear Maturation and Gene Expression of Immature Human Oocytes

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Abstract

Background: Vitrification of oocytes is a fast-freezing technique, which may affect the quality of human oocytes, and consequently affects the embryo development, pregnancy and birth. The aim of the current study was to investigate the consequences of in-vitro vitrification on maturation status of immature human oocytes, as well as expression levels of stress- and apoptosis-related genes.
Materials and Methods: The total of 213 human immature oocytes routinely discarded from assisted reproduction clinics were collected and divided into two groups including: (I) fresh germinal vesicle (GV) oocytes (n=106) matured in-vitro (fIVM) , and (II) GV oocytes (n=107) that were initially vitrified, then matured in in-vitro (vIVM). After 36 hours of incubation, the oocytes were evaluated for nuclear maturation and expression level of DNA methyltransferase (DNMT1), stress-related genes (Sod1 and Hsp70), and apoptosis-related genes (Bax and Bcl-2) by quantitative Real-Time PCR.
Results: Oocyte maturation rates were reduced in vIVM compared to fIVM oocytes (P=0.001). The expression of stress (Sod1 and Hsp70), and apoptosis-related genes (Bax and Bcl-2) in vIVM were significantly higher compared to fIVM group. Additionally, pro-apoptotic gene was up-regulated 4.3 times more than anti-apoptotic gene in vIVM oocyte. However, DNMT1 gene expression was reduced in vIVM oocyte (P = 0.047).
Conclusion: The low survival rate of vitrified in-vitro matured GV oocytes could definitely be explained by the alterations of their gene expression profile.

Keywords: Apoptosis; Oocytes; Vitrification

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Introduction

Vitrification of oocytes is a good method, which, unlike embryo cryopreservation does not conflict with ethical and legal issues (1). Moreover, oocyte cryopreservation provides a resolution for females who have lost their ovarian function due to surgery, chemo- or radiotherapy (2). The major problem associated with freezing of mature oocytes is the sensitivity of meiotic spindles to low temperature and cryoprotectants (3). To solve this problem, the germinal vesicle (GV) oocytes are used instead of mature oocytes. However, poor maturation, fertilization, and embryo development are the main disadvantages associated with cryostorage of immature oocytes (4).

It has been shown that vitrification as a stressor alters the gene expression and reduces the developmental competence of mouse zygotes (5). Furthermore, It is quite important to mention that, various factors in vitrification process, such as, high concentrations of cryoprotectants, freezing, and osmotic stress contribute to the alteration of gene expression pattern, and also the initiation of apoptosis; which threaten the viability and development of oocytes (6).

DNA methylation is an important epigenetic

mechanism, which plays critical roles in oocyte functions. Five different DNA methyltransferases (DNMTs) are responsible for adding a methyl group to the cytosine residues within the cytosine - phosphate-guanine (CpG) sites (7). DNMT1 is responsible for the sustainability of the methylation patterns during replication (8). Recently, Petrussa et al. showed that DNMT1 was constitutively present in the nuclei of human oocytes and embryos at all stages of preimplantation development until Day 7 after fertilization (9). In mouse, a desirable correlation has been found between aberrant genome-wide DNA methylation patterns,abnormal embryonic development, and preimplantation embryonic loss (10). According to the data, it has been suggested that, in human, reprogramming of DNA methylation during vitrification could be an important mechanism of 'fetal origin' diseases.

Vitrification causes stress; however, several enzymes eradicate the stress in the oocytes. Cu-Zn-superoxide dismutase Sod1 is expressed at a relatively high level in human and mouse at GV and Metaphase II stages of oocyte maturation (11). Sod1 is an antioxidant enzyme that converts the superoxide oxygen anion ($O2^-$) to $H2O2$, which is a less reactive oxygen species (12).

Vitrification-thawing could disrupt membrane asymmetry and cause apoptosis. The anti-apoptotic members of the Bcl-2 gene family play a vital role in regulating apoptosis, which promotes cell survival. Whereas, Bax is a pro-apoptotic molecule and accelerates the cell death (13).

The ultra-structural changes in the distribution pattern of the cortical granules (CGs), as well as cytoplasmic structural changes followed by vitrification of GV oocytes were reported in our previous study (14). Nonetheless, the influence of vitrification on the gene expression has been predominantly studied in mice; there are not a lot of studies in human oocytes until now with promising outcomes. Therefore, the aim of this study was to evaluate the effects of vitrification stress on maturation and expression patterns of apoptosis-related (Bax, Bcl-2), DNMT1 and stress-related genes (Sod1, HSP70,) of GV human oocytes.

Materials and methods

Patients

GV oocytes were obtained from patients who were referred to Yazd Research and Clinical Infertility Center (Yazd, Iran). The majority of patients had male factor infertility. It should be noted that we used the immature oocytes, which are usually discarded in stimulated cycles. This study was approved by the ethics committee of Shahid Sadoughi University of Medical Sciences and the patients who underwent intracytoplasmic sperm injection had signed a written consent form at Yazd Research and Clinical Infertility Center. The total of 97 females aged between 20-40 years were stimulated with the standard long stimulation protocol. Human chorionic gonadotropin (hCG; 10,000 IU) was injected 36 hours before oocyte collection. Then, the collected oocytes were cultured via in-vitro fertilization media (Vitrolife, Switzerland) and were placed in a 6% CO2 incubator (37 °C) for approximately 2-3 hours. Cumulus cells were removed by using 80 IU/ml hyaluronidase (Sigma, USA).

Table 1. Primers and probes used for RT-qPCR analysis.

Genes	Sequences (5'-3')	
Bax	Forward	CTG GAC AGT AAC ATG GAG
	Reverse	GCT GGC AAA GTA GAA AAG
	Probe	FAM-ATG ATT GCC GCC GTG GAC AC-BHQ1
Bcl-2	Forward	GGA TGA CTG AGT ACC TGA
	Reverse	CCG TAC AGT TCC ACA AAG
	Probe	FAM-CAG CCT CCG TTA TCC TGG ATC-BHQ1
HSP70	Forward	TGG AGG AGT TCA AGA GAA
	Reverse	GAT GGA CGT GTA GAA GTC
	Probe	FAM-ACA CAA GAA GGA CAT CAG CCA GA-BHQ1
Sod 1	Forward	CAG GGC ATC ATC AAT TTC
	Reverse	CCT TCA GTC AGT CCT TTA
	Probe	FAM-CCA CAC CTT CAC TGG TCC ATT ACT-BHQ1
Dnmt1	Forward	CGA AGT CAA ACC AAA GAA
	Reverse	GCT TCT TCT CAT CTT TCT C
	Probe	FAM-TCC ATC TTC GTC CTC GTC AGC-BHQ1
GAPDH	Forward	GCT GCT TTT AAC TCT GGT A
	Reverse	CCA TGT AGT TGA GGT CAA
	Probe	FAM-TGG ATA TTG TTG CCA TCA ATG ACC-BHQ1

Oocyte in-vitro maturation

Denuded oocytes were assessed for nuclear maturity, under the stereoscope (Olympus SZX10, Japan). In order to achieve a good maturation rate, high quality

oocytes (with fine homogeneous granular cytoplasm and compact granulosa cell layers) were selected and randomly divided into two groups; (I) fresh-IVM (fIVM; n=106) and (II) vitrified-IVM (vIVM; n=107). Denuded oocytes were transferred into maturation medium consisting of Ham's F10 (Biochrom, Germany) supplemented with 0.75 IU LH, 0.75 IU FSH (Ferring, Germany), and 40% of human follicular fluid (HFF). HFF was prepared as described previously (14). In order to separate blood and granulosa cells, HFF was centrifuged for 10 minutes, followed by 30 minutes inactivation at 56 °C, then the clear HFF was filtered by using a 0.22 µm filter; and finally, it was stored at -20 °C until use. The oocytes maturation was assessed using an inverted microscope (Nikon, Japan) after 36 hours incubation at 37 °C in 6% CO2; until the first polar body was observed.

Table 2. Demographic and clinical data of patients. Significant difference was not observed in the mean of age, etiology of infertility and total number of retrieved oocytes between fIVM and vIVM groups. The values inside parentheses represents (%).

Variable	Fresh-IVM (n=106)	Vitrified-IVM (n=107)	*P*-value
Age (years) (mean±SD)	29.3±5.9	32.2±5.5	0.34
Female factor infertility	49 (49.0)	51 (51.0)	0.11
Male factor infertility	47 (48.95)	49 (51.04)	0.11
Both (male and female infertility factors)	10 (52.63)	9 (47.36)	0.52

Vitrification and thawing of immature oocytes Based on the previously described protocol, vitrification of oocytes was performed at room temperature (15). Briefly, the oocytes were equilibrated for 10 minutes in a solution comprising of 7.5% ethylene glycol (EG) (Merck, Germany), 7.5% dimethyl sulfoxide (DMSO; Merck, Germany), and 20% human serum albumin (HSA) (Plasbumin, USA) in Ham`s F10; followed by incubation in vitrification for 60s in a solution of 15% EG, 15% DMSO, 0.5 M sucrose, and 20% HSA in Ham's F10. Oocytes were transferred into cryotops (Kitazato BioPharma, Japan) and stored in liquid nitrogen for a month. In order for thawing, at first, the cryotops were placed in thawing solution (1 M sucrose) (Sigma) at room temperature for 50-60 seconds; secondly, they were transferred into 0.5 M sucrose for 3 minutes; followed by 0.25 M sucrose for 3 minutes. All solutions were supplemented with Ham's F10 containing 20% HSA. The thawed GV oocytes were then rinsed 4-5 times in washing solution (Ham's F10 + 20% HSA). By the time thawing was done, the oocytes were transferred into IVM medium in 30µl droplets and were checked for survival, after an hour (15). Post-warming survival rate was evaluated by using morphological criteria, including the absence of overt cell degeneration, thick or distorted zona, expanded perivitelline space, and dark pronounced cytoplasm.

RNA isolation and reverse-transcription

In order to evaluate the expression levels of genes in the oocytes, 40 matured oocytes from each group lysed with RLT buffer (RNeasy Lyser Tissue, Qiagen, Germany) followed by RNA extraction. RNA extraction was performed by using an RNeasy Micro Kit (Qiagen, Germany) by following the manufacturer's instructions. The purity of samples was assessed by using A260/A280 nm ratio with expected value between 1.8 to 2.0. To remove genomic DNA, the RNA samples were treated with 1 U/µl of RNase-free DNaseI (Thermo, Lithuania) per 1 µg of RNA in the presence of 40 U/µl of ribonuclease inhibitor (Thermo, Lithuania) in 1 × reaction buffer with MgCl2, for approximately, 30 minutes at 37 °C. Furthermore, to perform DNaseI inactivation, 1 µl of 25 mM EDTA was added and incubated at 65 °C for 10 minutes. The purified RNAs were utilized for cDNA synthesis by using a RevertAid™ H minus First Strand cDNA Synthesis Kit (Thermo, Lithuania) and random hexamer primers, based on the manufacturer's instructions.

Real-Time Polymerase Chain Reaction

Gene expression was evaluated by quantitative real-time PCR. Primers and probes of the stress-related genes (Sod1 and Hsp70), apoptosis-related genes (Bcl-2 and Bax), DNMT1 and housekeeping gene (GAPDH) were designed using primer design software (Beacon Designer 7, USA), (Table 1). The specificity of design exon-exon spanning primers and probes were analyzed using NCBI Primer designing tool. Additionally, in order to confirm the specificity of primer, real-time PCR products were analyzed on 2% agarose gel. The real-time PCR mix in each tube included 2 µl of 10x PCR buffer, 2 µl of 50 mM Mgcl2, 0.4 µl of 10 mM dNTPs (Thermo, Lithuania), 2 µl of 10 fmol TaqMan probes that were labeled with FAM and BHQ1 in 5′ and 3′, respectively (Metabion, Germany), 0.2 µl of 5 U/µl Taq DNA polymerase (Cinnagen, Iran), 6.4 µl ddH2O, 1 µl of

each forward and reverse primers (10 pmol/µl), and 5 µl of single strand cDNA (12.5 ng/µl) in a final reaction volume of 20 µl. The real-time PCR reaction was carried out using a Rotor-gene Q real-time PCR System (Qiagen, Germany) based on the following procedure: 95 °C for 15s and 60 °C for 1 minute was repeated for 40 cycles. Non-reverse transcribed sample and no-template control were included in each run. The entire samples were tested in duplicates. The relative gene expression was calculated by REST mathematical model. For each gene, two-fold cDNA dilutions were prepared to generate standard curves and used to calculate the real-time PCR efficiencies [E=10(-1/slope)] (18).

Table 3. Comparison of maturation rates of human oocytes in fIVM and vIVM groups. The percentage of oocyte maturation in the fresh oocytes that were matured in-vitro without vitrification was significantly higher than the immature oocytes that were vitrified before maturation. The percentage of degeneration was also significantly higher in vIVM than in the fIVM group. †Values inside parentheses represents (%).

Variables	Fresh-IVM (n = 106)	Vitrified-IVM (n = 95)	*P*-value
Matured oocyte	91 (85.84)†	48 (50.52)	0.001
Degeneration	5 (4.71)	39 (41.05)	0.0001
Oocyte arrest	10 (9.43)	8 (8.42)	0.09

Statistical analysis

The differences in oocyte maturation rates in the groups were analyzed by using Student's t-test and Chi-squared test. The relative gene expressions in groups were compared by one-way analysis of variance (ANOVA) and post Tukey's test. The statistical analysis was performed using IBM SPSS Statistics software (version 21) and P-values of less than 0.05 were considered as the statistically significant.

Results

There were no significant differences in the mean of age, etiology of infertility, and total number of retrieved oocytes between fIVM and vIVM groups (Table 2). Additionally, There is no correlation between the vitrification and cause infertility.

The effect of vitrification on maturation of human oocytes

As it has been shown in Table 3, the percentage of oocyte maturation in the fresh oocytes that were matured in-vitro before vitrification were significantly higher than the immature oocytes that vitrified before maturation (P=0.001). The percentage of degeneration was also significantly higher in vIVM than in the fIVM group (41.05% vs. 4.71%; P = 0.0001).

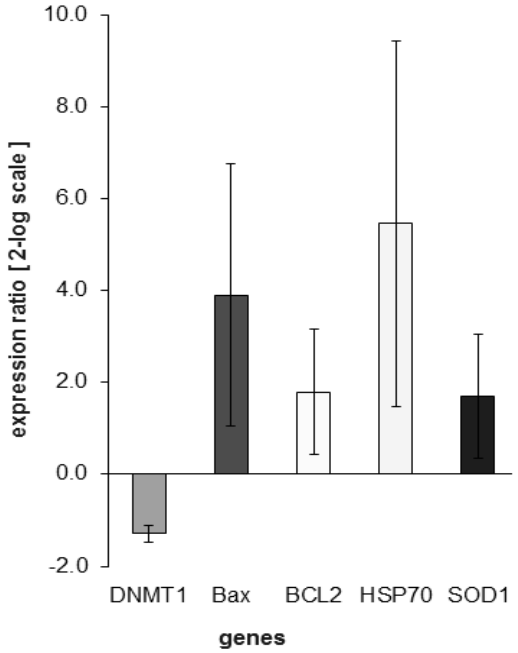

Figure 1. Relative mRNA expression of DNMT1, Bax, Bcl-2, HSP70 and Sod1 in vitrified-IVM oocytes. Taq-Man real-time PCR assay was used to quantitate relative gene expression. The entire samples were tested in duplicate.

The effect of vitrification on gene expression of human oocytes

In order to evaluate the effect of vitrification on the expression of the DNMT1, apoptosis-related (Bcl-2, Bax), and stress-related genes (Sod1, and HSP70); the expression patterns of these genes were analyzed in fIVM and vIVM groups. The results showed that expression of Bcl-2, Bax, Sod1 and HSP70 genes were significantly up-regulated in vitrified IVM group (Table 4). Similarly, DNMT1 was expressed in both groups, but showed reduced expression in vitrified IVM oocytes (P = 0.047). (Figure 1 and Table 4).

Table 4. Mean values of mRNA expression of DNMT1, Bax, Bcl-2, HSP70 and Sod1 in vitrified-IVM oocytes

Gene	Expression ratio (2-log scale)	P-value
DNMT1	1.29±0.17	0.047
Bax	3.90±2.84	0.008
BCL2	1.79±1.35	0.009
HSP70	5.45±3.98	0.007
SOD1	1.69±1.35	0.009

Discussion

During the process of cryopreservation, multiple factors such as hypoosmolarity and hyperosmolarity

stresses can lead to a variety of structural and biochemical cryoinjuries in oocytes (14, 17). This damage, in turn inversely affects the ability of oocytes to respond to signals to continue its development. Additionally, the highest concentration of cryoprotectants used in vitrification is toxic to cells (17). Cryoprotectants may not be completely removed from cells after warming up, and the remained cryoprotectants inside the cells may have the possibility to interact with cellular structures or molecules, which result in developmental arrest. Therefore, a quite notable weaknesses of oocyte cryopreservation in all current protocols is the compromised developmental competence of the oocyte, which mostly has been demonstrated in human and bovine oocytes (19,20). The results of the current study showed that the percentage of oocyte maturation in the oocytes that were matured in-vitro before vitrification was significantly higher, in comparison to immature oocytes, which were vitrified, then matured in-vitro (85.84% vs. 50/52% respectively). Cao et al. (2009) also reported that the oocyte maturation rates were significantly reduced in oocytes which were vitrified at GV stage and then matured in-vitro (50.8%), in comparison with oocytes which were first matured in-vitro and followed by vitrification (70.4%) (20). Son et al. reported that survival and maturation rates of immature human oocytes after freezing were 55.1%; and 59.3%, respectively (21). The differences between the results of various studies are probably due to the differences in the freezing methods and culture medium. Multiple factors including media composition, growth factors or hormonal supplements, and the origin of the oocytes (stimulated vs. unstimulated cycles) affect the percentages of oocyte maturation following IVM (22).

The results showed that the percentage of degeneration in vIVM was significantly higher than (41.05% vs. 4.71%; P = 0.0001). Men et al. (2003) demonstrated that the degeneration mechanism of cryopreserved oocytes is apoptosis (6). It has been shown that inclusion of an apoptosis inhibitor (caspase I inhibitor V) in the cryopreservation solution was effective to reduce post-cryopreservation apoptotic degeneration of a canine renal cell line (23). Therefore, the assessment of apoptotic gene expression can be considered as an effective tool to evaluate the viability of oocytes following vitrification. Bcl-2 family is apoptosis regulator, which controls the permeabilization of the mitochondrial outer membrane. They are either pro-apoptotic such as Bax and BAD, or anti-apoptotic including Bcl-2 and Bcl-xL. Dhali et al. showed that there is a strong association between compromised development competency and alteration in expression level of Bax, Bcl-2, and p53 genes in the vitrified embryos (24). In immature bovine oocyts, the transcription levels of Bcl-2, Fas, FasL and Bax increased after a process of vitrification and warming (25). In this study, although the expression of apoptosis-related genes was significantly up-regulated in vIVM and fIVM groups; the results revealed that the pro-apoptotic (Bax) mRNA level increased more than 14 times in vitrified oocytes, compared to the fresh oocytes. In fact, the Bcl-2 gene expression increased only 3.46 times. The ratio of Bcl-2 to Bax may be useful for measuring the oocytes tendency towards either survival or apoptosis. The results of this study showed that the ratio of pro-apoptotic to anti-apoptotic mRNA, was 4.3 times in vIVM oocyte.

It has been noticed that vitrification could induce oxidative stress in oocytes following warming step. Cumulus cells have a critical role in protecting oocyte against oxidative stress (26). Turathum et al. (2010) reported that the expression levels of HSP70 and Sod1 genes in control and vitrified-warmed canine oocytes could be comparable with cumulus cells (27). The focus of the present study was on the effect of vitrification on oocytes without cumulus cells. The results showed that expression of stress genes (Sod1 and HSP70) was higher in vIVM group compared to the fIVM group (P = 0.009, and P = 0.007, respectively). Furthermore, Habibi et al. reported that in vitrified mouse oocytes Sod1 gene expression was higher than the controls (28).

Previous studies on animals revealed that vitrification could influence the epigenetic makeup of oocytes. Vitrification of pig oocytes induces changes in histone H4 acetylation and histone H3 lysine 9 methylation (29). Additionally, vitrification significantly decreases the expression of DNMT1 mRNA in mouse metaphase II oocytes (30, 31). Our results showed that the expression level of DNMT1 got reduced in vIVM group. Reduction of DNMT1 gene expression may cause alteration in the patterns of DNA methylation, resulting in disruption of gene expression, genomic imprinting, and genome stabilization, which can lead to cell death. The expression of DNMT1, seems to be more disturbed in poor quality (fresh and cryopreserved) embryos, in comparison to good quality cryopreserved embryos and delayed fresh embryos (9). It should be noted that, apart from limitations in sample collection, the gene expression analysis on limited number of oocytes was a challenge, due to the small amount of RNA which was extracted.

Conclusions

Vitrification, apart from all drawbacks on oocyte ultra-structure, can affect the oocyte mRNA content.

In this study, for the first time, it was shown that expression of stress and apoptosis-related genes increased post vitrification-warming of human immature oocytes. Therefore, low survival rate of vitrified in-vitro matured oocyte could be explained by the alterations of their gene expression profile. According to our results, the use of vitrified immature human oocytes should be reconsidered, more studies have to be done to confirm these findings though.

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Author contributions

ShA has participated in the all parts of study. HA designed the study and participated in draft preparation. KhMA has contributed to study design and manuscript preparation. YF participated in designing the experiments, data analysis and paper preparation.

Conflicts of Interest

The authors declare that there is no financial or other conflict of interests related to this paper.

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