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Human Ovarian Tissue Vitrification/Warming Has Minor Effect on the Expression of Apoptosis-Related Genes

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ABSTRACT

Background: In this study, we evaluated the incidence of apoptosis at the ultrastructural levels and expression of some apoptosis-related genes in vitrified human ovarian tissue just after warming. **Methods:** Human ovarian tissue biopsies from 23 women after caesarean section were transported to the laboratory within 2 hours, and then they were cut into small pieces. Some pieces were vitrified and warmed and the other samples were considered as control. Apoptosis was assessed by a transmission electron microscope and also by molecular analysis of pro-apoptotic (*Fas*, *FasL*, *Bax*, *p53*, *caspase8*, and *caspase3*) and antiapoptotic (*Bcl-2* and *BIRC5*) genem RNA levels using real-time RT-PCR before and after vitrification. **Results:** No sign of apoptosis was shown ultrastructurally in vitrified samples. The level of *FasL*, *Bcl-2*, *Bax*, *p53*, and *caspase3* mRNA and *Bax:Bcl-2* ratio were similar in non-vitrified and vitrified groups; however, the expression of *Fas* and *caspase8* genes was higher and *BIRC5* was lower in vitrified samples compared to non-vitrified group ($P<0.05$). **Conclusion:** The fine structure of human vitrified ovarian tissue was well preserved; moreover, vitrification was shown to affect the expression of some apoptosis-related genes. However, additional study is needed to confirm this observation. *Iran. Biomed. J. 17 (4): 179-186, 2013*

Keyword: Vitrification, Apoptosis, Gene expression, Ovary, Humans

INTRODUCTION

There are two methods for cryopreservation of human ovarian tissue: conventional slow freezing and cryopreservation by high concentration of cryoprotectant and direct plunging into liquid nitrogen (vitrification). The first attempt for cryopreservation of human ovarian tissue carried out by Zhang *et al.* [1] using ultrarapid freezing with DMSO and sucrose. To evaluate and compare the outcome of different cryopreservation protocols (safety of technique), some investigations were focused on the assessments of the normality of follicles and the incidence of cell death after thawing or warming [2-5]. Apoptosis may occur not only in normal ovarian tissue [6] but also during cryopreservation [7, 8].

The viability of ovarian follicles and the incidence of apoptosis were assessed after warming of vitrified human ovarian tissue, and there were some controversy in this regards. Different studies have shown the vitrification of human ovarian tissue provides similar results compared to the conventional slow-freezing technique [8-11]. Some studies have reported some cryodamage in ovarian tissue [4, 5]. Gandolfi *et al.* [4] have shown that vitrification causes an extensive damage to preantral follicles of ovarian tissue. The efficiency of vitrification as a cryopreservation method for human ovarian tissue has been supported by several studies [7, 10-17].

In different studies, Xiao *et al.* [13] and Chang *et al.* [7] have recently shown the fewer TUNEL-positive cells in vitrified human ovarian tissue as compared with fresh or slow-cooled tissue. However, Zhou *et al.* [18] demonstrated that the incidence of apoptotic cell in vitrified ovarian tissue was significantly higher than fresh tissue.

Recently, we compared the incidence of apoptosis in human ovarian tissue by two cryopreservation methods not only after warming but also after 24 hours *in vitro* culture. In addition, we observed no sign of apoptosis in both cryopreservation groups regarding to different methods for apoptosis assessment, such as DNA laddering, TUNEL assay, and transmission electron microscopy [8]. However, it has not been reported so

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far whether vitrification of human ovarian tissue induces the incidence of apoptosis at the molecular level.

According to our knowledge, there is poor report about the evaluation of apoptosis at the molecular levels (mRNA) after vitrification of human ovarian tissue, even there is very limited attention to another mammalian models in this regard [19, 20]. Mazoochi *et al.* [19] demonstrated some changes in expression of genes related to apoptosis in mouse vitrified ovarian follicles after *in vitro* culture [19]. There are at least two broad pathways that lead to ovarian tissue apoptosis: intrinsic pathway that involved several apoptotic genes, such as *Bcl-2*, *Bax*, p53, and *BIRC5* [21-24] and extrinsic pathway such as *Fas/FasL* system [25, 26]. In both pathways, signaling results in the activation of a family of cysteine proteases such as *caspase 8*, and *caspase 3* that act in a proteolytic cascade to remove the dying cell [27, 28]. Among these genes, some are antiapoptotic (*Bcl-2* and *BIRC5*) and some are pro-apoptotic (*Fas*, *FasL*, *Bax*, *p53*, *caspase8*, and *caspase3*).

Therefore, the present study was the first attempt to evaluate the expression of the apoptosis-related genes (*Fas*, *FasL*, *Bcl-2*, *Bax*, *p53*, *BIRC5*, *caspase 8*, and *caspase 3*) in vitrified-warmed human ovarian tissue in comparison with non-vitrified using real-time RT-PCR.

MATERIALS AND METHODS

Reagents and materials were obtained from Sigma-Alderich (Germany) except mentioned otherwise.

Ovarian tissue collection. An informed consent was given by 23 women aged between 24-35 years old (median 28) under a protocol approved by the Ethics Committee of the Faculty of Medical Science of Tarbiat Modares University (Ref. No. 5274856), Tehran, Iran. Ovarian cortical tissue fragments of approximately 5 × 5 × 1 mm were obtained from women undergoing elective caesarean sections. Then, they were transported to the laboratory within 1-2 h with pre-warmed and equilibrated Leibovitz'sL-15 medium supplemented with 10 mg/ml human serum albumin, 100 IU/ml penicillin and 100 µg/ml streptomycin. The ovarian cortexes were cut into small pieces approximately 2.5 × 1 × 1 mm under a sterile laminar hood with transfer medium. Then, these fragment tissues were randomly divided into non-vitrified and vitrified groups.

Vitrification and warming procedure. The tissues were vitrified according to the protocol described by Salehnia *et al.* [29] with some modifications. The vitrification solution was made of Ethylene glycol, Ficoll and Sucrose that named EFS40% containing 40% ethylene glycol (v/v), 30% Ficoll 70 (w/v), and 1 M sucrose supplemented with 0.21% human serum albumin instead of BSA. The human ovarian tissues were equilibrated in three changes of vitrification solutions for 5 minutes. Following the last incubation, individual tissue samples were placed into aseptic cryovials containing 100 µl vitrification solutions. The tubes were then put under the nitrogen vapor for 30 s finally immersed and stored in liquid nitrogen at least for two months. The tissues were thawed by immersing the vials in 37°C bath water with gentle agitation until melting the samples. Then, they were washed serially in 1, 0.5, 0.25 M sucrose and phosphate buffer I (PBI) containing 10 mg/mL human serum albumin at room temperature for 5 min. The samples were equilibrated in McCoy's culture media for 30 min.

Transmission electron microscopy. For assessment of nuclear fragmentation regarding to apoptosis or cell death, the tissue samples including non-vitrified (n = 3) and vitrified (n = 3) human ovarian tissues in 2.5% glutaraldehyde in PBS (pH 7.4) for 2 hours and post-fixed with 1% osmium tetroxide in the same buffer for 2 hours. After dehydration in an ascending series of ethanol, specimens were placed in acetone and embedded in epoxy resin. Semithin sections were stained with toluidine blue and studied under light microscopy. The thin sections were stained with uranyl acetate and lead citrate using a transmission electron microscope (Zeiss, 911, Germany).

RNA extraction and cDNA synthesis for molecular assessment. Total RNA was extracted from non-vitrified (n = 3), vitrified (n = 3), and apoptosis-induced human ovarian tissue groups using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The RNA concentration was determined by spectrophotometry and adjusted to a concentration of 250 ng/ml. Using oligo dT, RNA was reverse transcribed by Moloney murine leukemia virus reverse transcriptase. Using specified primers (Table 1), the *Fas*, *FasL*, *Bcl-2*, *Bax*, *p53*, *BIRC1*, *caspase8*, and *caspase3* genes were amplified. *GAPDH* gene was used as an internal control.

Real-time RT-PCR. After extraction of total RNA and cDNA synthesis, one-step RT-PCR was performed on Applied Biosystems (UK) real-time thermal cycler according to QuantiTect SYBR Green RT-PCR kit (Applied Biosystems, UK, Lot no:1201416). Prior to the quantitative analysis, optimization procedures were performed by running real-time RT-PCR with or

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Table 1. Oligonucleotide primers

Accession numbers	Gene	Primer sequence	PCR product size (bp)
NC_000012.11	*GAPDH*	Forward:5`CTGGGCTACACTGAGCACC 3` Reverse:5`AAGTGGTCGTTGAGGGCAATG3`	101
NC_000010.10	*Fas*	Forward: 5`TGAAGGACATGGCTTAGAAGTG 3` Reverse:5`GGTGCAAGGGTCACAGTGTT3`	118
NC_000001.10	*FasL*	Forward: 5´GCAGCCCTTCAATTACCCAT 3` Reverse:5`CAGAGGTTGGACAGGGAAGAA3`	101
NC_000018.9	*Bcl-2*	Forward:5`TTGCTTTACGTGGCCTGTTTC3` Reverse:5`GAAGACCCTGAAGGACAGCCAT3`	94
NC_000019.9	*Bax*	Forward: 5`CCCGAGAGGTCTTTTTCCGAG3` Reverse:5`CCAGCCCATGATGGTTCTGAT3`	155
NC_000017.10	*p53*	Forward: 5´GAGGTTGGCTCTGACTGTACC3` Reverse:5`TCCGTCCCAGTAGATTACCAC3`	133
NC_000017.10	*BIRC5*	Forward:5`AGGACCACCGCATCTCTACAT3` Reverse:5`AAGTCTGGCTCGTTCTCAGTG 3`	118
NC_000002.11	*Caspase 8*	Forward: 5`ATTTGCCTGTATGCCCGAGC 3` Reverse:5`CCTGAGTGAGTCTGATCCACAC3`	105
NC_000004.11	*Caspase 3*	Forward: 5´AGAGGGGATCGTTGTAGAAGTC 3` Reverse:5`ACAGTCCAGTTCTGTACCACG3`	81

without template to verify the reaction conditions, including the annealing temperatures of the primers and specific products. For target sequence amplifications, 100 ng (5 µl) of RNA was used per 20 µl reaction volume. After completing the PCR run, melt curve analysis was used to confirm the amplified product. For each sample, the reference gene (*GAPDH*) and the target genes were amplified in the same run. Standard curves were obtained using the logarithmic dilution series of total RNA. Real-time thermal condition included holding step at 95ºC for 5 min, cycling step at 95ºC for 15 s, 58ºC for 30 s, and 72ºC for 30 s, and it was continued by a melt curve step at 95ºC for 15 s, 60ºC for 1 min, and 95ºC for 15 s. Then, relative quantification of target genes was determined using the Pfaffl method [30]. The real-time RT-PCR experiments were repeated three times.

Statistical analysis. Statistical analysis was carried out with SPSS 19.0 software. Quantitative variables were expressed as mean ± SD. The results of real-time RT-PCR were compared by one-way ANOVA test and post hoc Turkey's test ($P \leq 0.05$).

RESULTS

The follicular morphology of vitrified and non-vitrified ovarian tissue in semithin sections. The morphology of human ovarian tissue after vitrification and warming were well preserved. No morphological sign of apoptosis, including pyknotic and fragmented nuclei and cytoplasmic vacoules in ovarian follicles and stromal cells were observed in the follicles after semithin section preparation (Fig. 1). There were some small primordial and primary follicles within these tissues.

Ultrastructural observation. The ultrastructure of nucleus of oocyte and follicular cells of primary follicles in vitrified group were well preserved and seemed to be similar to the non-vitrified group (Fig. 2). The oocyte had a euchromatic nucleus at the germinal vesicle stage. The follicular cells in both groups had normal ultrastructure. No nucleus fragmentation, shrinkage of cell membrane, and vacuoles were shown within cytoplasm of oocyte and follicular cells. The euchromatic nuclei showed peripheral aggregates of heterochromatin. However, the ultrastructure of stromal cells within both vitrified and non-vitrified

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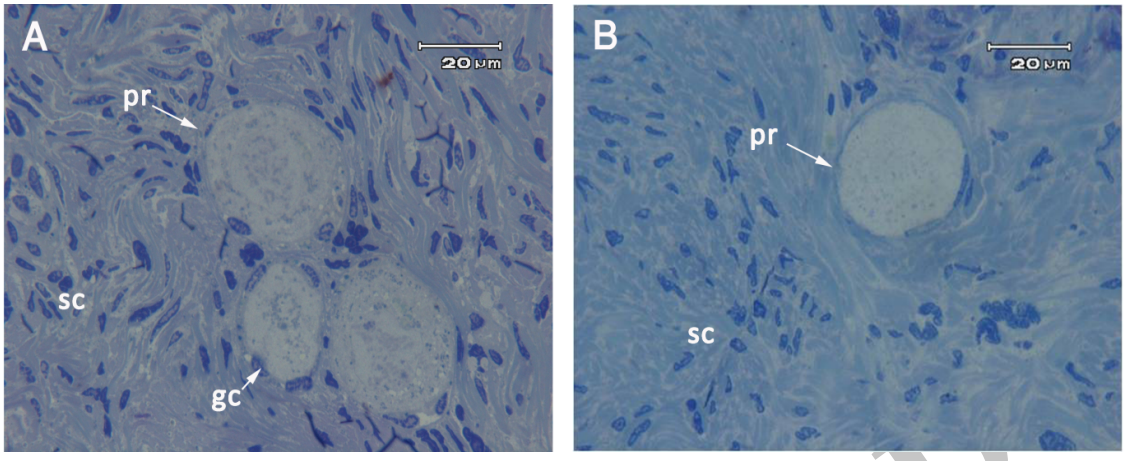

Fig. 1. Semithin section of human ovarian tissue, Non-vitrified **(A)** and vitrified **(B)** groups. There were some primordial follicles (pr) with normal morphology. gc, granulosa cell; sc: stromal cell

tissue has been shown in Figure 3. There were not any sign of fragmentation of nucleus within the stromal cells.

Expression of apoptosis-related genes in non-vitrified and vitrified human ovarian tissue. The mRNA levels of several apoptosis-related genes, including *Fas*, *FasL*, *Bax*, *p53*, *caspase8*, and *caspase3*, *Bcl-2*, and *BIRC5* genes were evaluated in non-vitrified and vitrified human ovarian tissue by real-time RT-PCR. The expression ratio of the target gene to housekeeping (*GAPDH*) gene in vitrified and non-vitrified samples has been shown in Figure 4. As the results demonstrated, among the studied genes, the expression of *FasL*, *Bcl-2*, *Bax*, *p53*, and *caspase3* was not changed significantly in both groups. The level of Fas and *caspase8* mRNA was significantly higher in the vitrified group compared with the non-vitrified sample, but the *BIRC5* mRNA level was significantly lower in the vitrified group in comparison with its control (P<0.05).

DISCUSSION

The vitrification method is a simple alternative method for cryopreservation of human ovarian tissue. Results of the present study showed no ultrastructural changes, including nuclear fragmentation, mitochondrial disruption, shrinkage of cell membrane, and sytoplasmic vacuolein the oocyte as well as follicular and stromal cells after vitrification and warming of ovarian tissue. On the other hand, there were not any apoptotic changes in ovarian cells at the ultrastructural level in vitrified samples compared to the control group. Moreover, our observation confirmed the safety of vitrification procedure using EFS40 for human

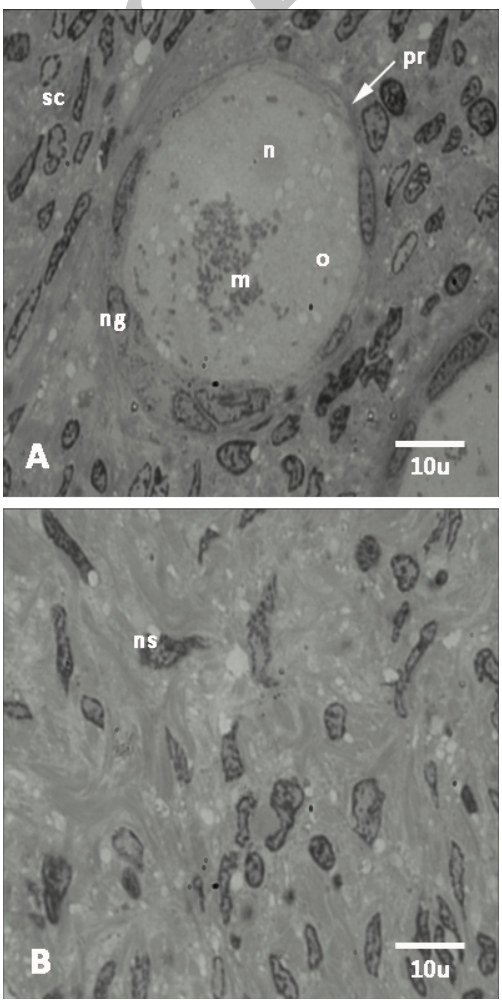

Fig. 2. Transmission electron micrographs of non-vitrified ovarian tissue. Ultrastructure of primordial follicles (pr) **(A)** and stroma **(B)**. n, nucleus; o, ooplasm; m, mitochondria; ng, granulosa cell nucleus; sc, stromal cell; ns, stromal cell nucleus

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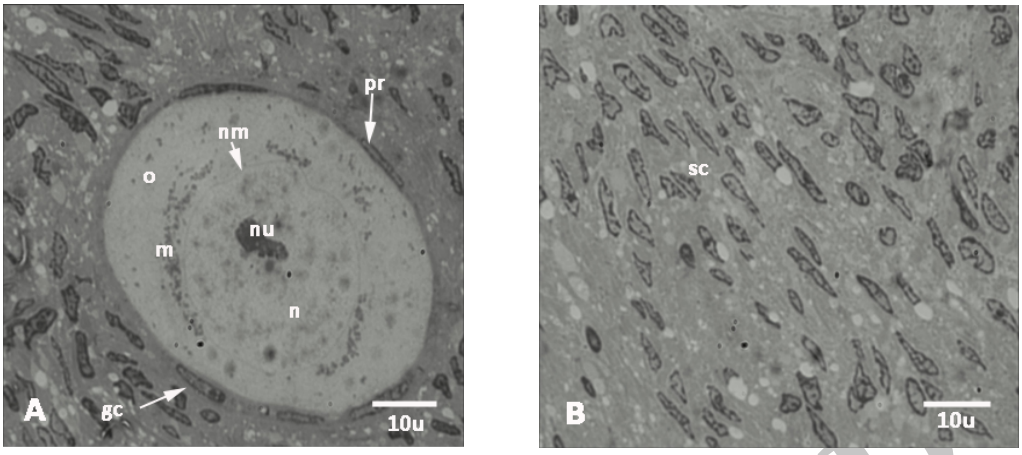

Fig. 3. The fine structure of human ovarian tissue. Primordial follicles **(A)** and stroma **(B)** of vitrified-warmed group. Pr, primordial follicle; nu, nucleolus; nm, nuclear membrane; n, nucleus; o, ooplasm; m, mitochondria; gc, granulosa cell; sc, stromal cell. The ultrastructure of nucleus of oocyte and granulosa cells of primary follicle was well preserved and was similar to the non-vitrified group. The oocyte had a euchromatic nucleus at the germinal vesicle stage. No nucleus fragmentation, shrinkage of cell membrane and vacuoles within cytoplasm of oocyte and granulosa were seen.

ovarian preservation based on morphological and ultrastructural study [8].

Also, the results of our previous study showed no significant changes in chromatin condensation in nucleus of different types of cells within human ovarian tissue which subjected to vitrification or ultrarapid cryopreservation [8]. However, similar conclusion was published by some other researchers in this regard. Keros *et al.* [10] compared vitrification and slow programmed freezing of human ovarian tissue. They revealed by electron microscopy that the ovarian stroma was significantly better preserved after vitrification compared to slow freezing.

Sheikhi *et al.* [14] have shown that vitrification is an excellent method to cryopreserve ovarian tissue. They did not observe any differences in the ultrastructure of oocytes between non-vitrified and vitrified tissues. Moreover, they have recently demonstrated that the fine structure of oocytes, granulosa, and ovarian stromal cells in vitrified human ovarian tissue are well preserved by electron microscopic analysis. They used ethylene glycol as a permeating cryoprotectant and compared it with different solutions [16].

Wang *et al.* [11] showed that the primordial follicles in vitrified human ovarian tissues by needle immersed vitrification were well preserved and the ultrastructure of the stromal cells was better preserved in vitrified group than the slow-freezing or the dropping vitrification. In contrast, Zhou *et al.* [18] compared different vitrification procedures using several cryoprotectant solutions for human ovarian tissue. Their ultrastructural observation showed irregularly shaped or swollen mitochondria in the cytoplasm of both oocytes and follicular cells. However according to our knowledge, there was not any report regarding to apoptotic changes of human ovarian tissue after vitrification.

In the other part of this study, we evaluated, for the first time, the expression of some apoptosis-related genes in vitrified human ovarian tissue. Our observation showed no significant difference between the mRNA levels of some pro-apoptotic genes, including *FasL*, *Bax*, *p53*, and *caspase3* and antiapoptotic gene *Bcl-2* in non-vitrified and vitrified samples. We concluded that the vitrification of human ovarian tissue using EFS40 did not induce apoptosis cell death at the gene expression levels. According to this panel of genes, we suggested that after vitrification none of intrinsic and extrinsic pathways of apoptosis were induced.

There are two mechanisms for ovarian tissue apoptosis: one mechanism is triggered by the binding of death molecules to cell surface receptors such as *Fas/FasL* system (cell death signaling or extrinsic pathway). The other one (intrinsic or mitochondrial pathway of apoptosis) is generated by the signals occurring within the cell, mitochondria and *Bcl-2* family members (*Bcl-2 and Bax)* [31-33]. In both pathways, signaling resulted in the activation of a family of cysteine proteases named caspases, that act in a proteolytic cascade to remove the degenerating cell [34]. The *p53* protein is an antiproliferative transcription factor that controls genomic integrity by inducing cell cycle arrest or apoptosis. This protein is expressed in the apoptotic granulosa cells of atretic follicles [35]. Parallel with our observation, Hussein *et al.* [36] demonstrated *p53* expression was not altered after slow freezing of human ovarian tissue.

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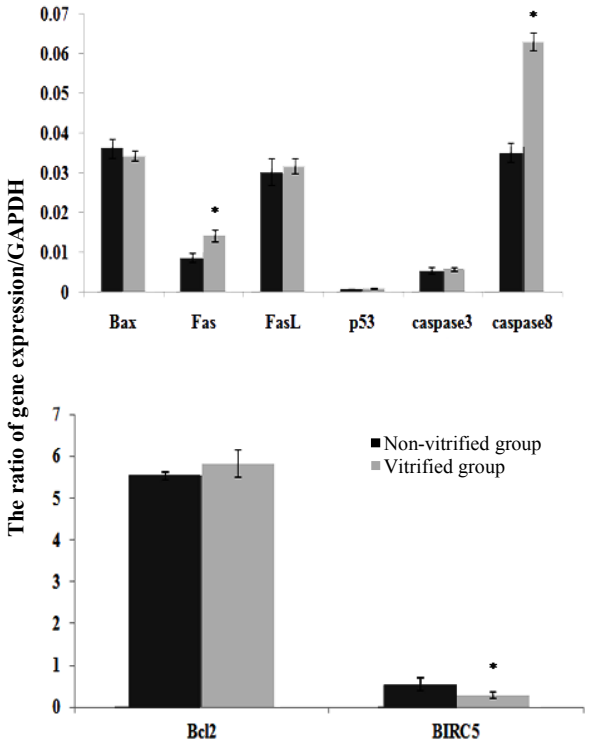

Fig. 4. Comparison of expression of apoptosis-related genes to *GAPDH* in non-vitrified and vitrified human ovarian tissue. The ratio of genes expression of *Bax*, *Fas*, *FasL*, *p53*, *caspase 3*, and *caspase 8* to *GAPDH***(A)** and the ratio of *Bcl-2*, *BIRC5* to GAPDH **(B)** using real-time RT-PCR in non-vitrified and vitrified groups. *Significant differences between groups (P<0.05).

Bcl-2 is an antiapoptotic and *Bax* is a proapoptotic factor. *Bax* can suppress the ability of *Bcl-2* to block apoptosis. Ratio of *Bax:Bcl-2* can influence the ability of a cell to respond to an apoptotic signal. The expression level of *Bax:Bcl-2* in vitrified group was the same as control group. We concluded that vitrification did not change the susceptibility of the ovarian tissue to apoptotic signals. When *Bcl-2* is in excess the cells are protected, and when *Bax* is in excess, the cells are susceptible to apoptosis [37].

A similar report was published by Depalo *et al.* [38] that compared the *Bax/Bcl-2* ratio in fresh and slow frozen human ovarian tissue samples.

In other part of this study, the molecular analysis showed the higher expression of proapoptotic genes (*Fas* and *caspase8*) in parallel with lower expression of antiapoptotic gene (*BIRC5*) in vitrified samples compared to the control group. Moreover, *caspase8* expression as an initiator caspase was increased in vitrified ovarian tissue samples, but it did not result in high expression of *caspase3*. On the other hand, the expression level of *caspase8* is not sufficient to activate other effector caspases in apoptosis.

In other point of view we suggested that these changes may be reversible and do not influence the viability of ovarian tissue cells, or these different mRNA levels have not any effect on the *in vitro* or *in vivo* subsequent survival of tissue and their follicular development. Confirmation of these suggestions needs more study to analyze the incidence of apoptosis after short- and long-term culture by some complementary techniques.

According to our knowledge, there is not any report regarding the evaluation of cell death at the molecular level in human and animal models after vitrify-cation/warming. However, the similar pattern of apoptosis gene expression has been reported by Mazoochi *et al.* [19] during *in vitro* culture of isolated follicles derived from vitrified mice ovarian tissue. They showed that the expression of some apoptotic related genes (*Fas* and *BIRC5*) was changed, and that of other genes (*p53*, *Bcl-2*, *Bax*, and *FasL*) did not affect after *in vitro* culture of isolated follicles derived from vitrified mouse ovarian tissue.

In conclusion, no signs of apoptosis were observed at the ultrastructural levels in the vitrified and warmed human ovarian tissue, and the follicles and stromal cell integrity were well preserved. In spite of some changes in *FasL*, *BIRC5* and *caspase8* gene expression, the vitrification did not have any effect on the expression of *FasL*, *Bcl-2*, *Bax*, *p53* and *caspase3* genes. Therefore, additional studies are needed to confirm accuracy of these observations after a long-term culture or transplantation of human ovarian tissue.

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