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Over Vitrifikasyonunun Mitokondriyal Füzyon (MFN-1, MFN-2 ve OPA-1), Fisyon (DNM-1), Mitofaji (PARKIN, PINK-1) ve Transport (MIRO-1, MILTON) Proteinleri Üzerindeki Etkileri

The Effects of Ovarian Vitrification on Mitochondrial Fusion (MFN-1, MFN-2 and OPA-1), Fission (DNM-1), Mitophagy (PARKIN, PINK-1) and Transport (MIRO-1, MILTON) Proteins

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Öz

Giriş ve Amaç: Ovaryan kriyoprezervasyonu, yardımcı üreme teknolojilerinde doğurganlığın korunması için yararlı bir alternatiftir. Vitrifikasyon prosedüründeki birçok ilerlemelere rağmen bu teknik hala deneysel olarak kabul edilmektedir. Bu nedenle bu çalışmada, vitrifikasyon sonrası over dokularında mitokondriyal füzyon (*MFN1, MFN2* ve *OPA1*), fisyon (*DRP1*), mitofaji (*PARKIN, PINK1*) ve transport (*MIRO-1, MILTON*) proteinlerinin ifadelerini qPCR tekniği ile araştırmayı amaçladık.

Gereç ve Yöntemler: Vitrifikasyon sonrası mitokondriyal dinamikleri araştırmak için, overler 6-8 haftalık sağlıklı dişi farelerden (No: 12) alındı ve vitrifikasyon ve kontrol gruplarına ayrıldı. Vitrifikasyon, etilen glikol, dimetilsülfoksit ve sukroz kullanılarak gerçekleştirildi. Kontrol ve vitrifikasyon gruplarındaki overlerden total RNA izolasyonu yapıldıktan sonra hedef genlerin ifade oranlarını belirlemek için qPCR tekniği kullanıldı. Hedef genlerin relatif gen ifadeleri $2^{-\Delta\Delta Ct}$ yöntemine göre değerlendirildi.

Bulgular: Histolojik değerlendirme kontrol grubundaki overlerin normal morfoloji gösterdiği, vitrifikasyon grubundaki overlerin ise doku bütünlüğünün bozulduğunu; bazı foliküllerin dejenere olduğunu ve granüloza hücrelerinin antruma döküldüğünü ortaya koydu. qPCR sonuçlarımıza göre vitrifikasyon grubunda kontrol grubuna kıyasla dış membran füzyon proteini *MFN1* gen ifadesinin 1,12 kat azaldığı ve iç membran proteini olan *OPA-1*'in ifadesinin 1,36 kat arttığı saptandı. Mitokondriyal fisyon proteini *DRP-1* gen ifadesinin vitrifikasyon grubunda 1,20 kat arttığı bulundu. Mitofaji proteinleri olan *PINK-1* ve *PARKIN* gen ifadelerinin vitrifikasyon grubunda sırasıyla 1,34 ve 3,75 kat azaldığı tespit edildi. Kontrol grubuna göre karşılaştırıldığında transport proteinlerinin; *MIRO-1* gen ifadesinin 1,16 kat azaldığı ancak *MILTON* (*TRAK-1*) gen ifadesinin 2,28 kat arttığı saptandı.

Sonuç: Mitokondriyal dinamikler ile ilişkili gen ifadelerindeki değişimler, ovaryan vitrifikasyonu sırasında mitokondriyal fonksiyonda bir azalmaya yol açabilir ve oosit maturasyonu ve embriyo gelişimi potansiyelini azaltabilir.

Anahtar Kelimeler: Mitokondri, Mitokondriyal Fisyon, Mitokondriyal Füzyon, Mitofaji, Ovaryan Vitrifikasyon

Abstract

Objective: Ovarian cryopreservation is a useful alternative for fertility preservation in assisted reproductive technologies. In spite of many advances in the vitrification procedure, this technique is still considered experimental. Therefore in this study, we aimed to investigate the expressions of mitochondrial fusion (*MFN1, MFN2* and *OPA1*), fission (*DRP1*), mitophagy (*PARKIN, PINK1*) and transport (*MIRO-1, MILTON*) proteins in ovarian tissues by qPCR technique after vitrification.

Materials and Methods: To investigate the mitochondrial dynamics after vitrification, the ovaries were recovered from 6-8 week old healthy female mice (No: 12) and were divided into vitrification and control groups. Vitrification carried out using ethylene glycol, dimethylsulfoxide and sucrose. After total RNA isolation from ovaries in control and vitrification groups, qPCR technique was performed to determine the expression rate of target genes. The relative gene expressions of the target genes were evaluated according to $2^{-\Delta\Delta Ct}$ method.

Results: Histological evaluation revealed that ovaries in the control group were shown normal morphology while the tissue integrity of the ovaries in the vitrification group is disrupted, some follicles are degenerated and granulosa cells were shed into antrum. According to our qPCR results, outer membran fusion proteins *MFN1* gene expression decreased 1.12 fold and inner membran protein *OPA-1* increased 1,36 fold in the vitrification group compared the control group. The mitochondrial fission protein *DRP-1* gene expression increased 1.20 fold in the vitrification group. The mitophagy proteins *PINK-1* and *PARKIN* genes expressions decreased 1.34 and 3.75 fold respectively in the vitrification group. The transport proteins; *MIRO-1* gene expression decreased 1.16 fold but *MILTON* (*TRAK-1*) gene expression sharply increased 2,28 fold compared the control group.

Conclusion: The alternation of the mitochondrial dynamics related gene expressions may lead a decrease in the mitochondrial function during the ovarian vitrification and may reduce the potential of oocyte maturation and embryo development.

Keywords: Mitochondrion, Mitochondrial Fission, Mitochondrial Fusion, Mitophagy, Ovarian Vitrification.

1. Introduction

Chemotherapy and radiotherapy are used for cancer treatment, however these therapies often cause female reproductive dysfunction. Advances in cancer treatment have led to a considerably higher survival rate, including for prepubescent girls and women of reproductive age, so there is also an increase in oocyte and ovarian freezing desires of women who want to have children at a later age

[1]. Ovarian tissue freezing (OTF) and storage is a new technique using to protect fertility of women. However OTF is difficult from oocyte and blastocyst freezing, because of the presence of a large number of cells and deeper follicles [2]. In addition this, high doses of cryoprotectants increase tissue toxicity, the large size of the tissue prolongs the penetration time of the cryoprotectants. Two common methods are used for OTF. In the slow freezing technique, the follicular pool and stromal cells are generally losses. The other technique is vitrification, rapid cooling technique, which supply good results OTF like as blastocyst and oocyte freezing [3]. The lack of ice crystals during the application of the vitrification technique protects the cells from mechanical damage. It has also been shown that this technique provides better preservation of the morphological integrity of stromal cells compared to slow freezing [4]. During the cryopreservation process; both physical and chemical changes occur in the cells. Cellular membranes, cellular skeleton and mitochondrial damage were observed after cryopreservation. In addition this, mitotic spindle damage, karyotype changes, exocytotic excretion of cortical granules, swelling in the flat endoplasmic reticulum, and mitochondrial damage in the cytoplasm of oocytes occur after ovarian freezing and thawing [5].

Mitochondria are the major ATP production organelles of cells and serve as key regulators of multiple vital cellular processes, including apoptosis, calcium homeostasis, and the generation of ATP via the metabolic pathway known as oxidative phosphorylation (OXPHOS). The energy is required during the nuclear and cytoplasmic maturation of oocytes and also preimplantation embryo development. It is known that, the mitochondrial damage cause failure of oocyte development and maturation and lead to abnormal embryo development [6-8]. It is suggested that, during the vitrification, oocyte cytoplasm shrinks and then swelling affects the mitochondrial distribution, however its not known completely the effect of vitirfication on mitochondria [9]. Mitochondria are dynamic organelles which have its own mechanism of hemostasis. With its own proteolytic system, damaged outer membrane proteins can be removed. An important feature of mitochondrion is that the damaged mitochondrial fragments can be removed by fission and fusion processes. Mitochondria able to change their shape by undergoing fusion to generate elongated interconnected mitochondrial networks by mitochondrial fusion proteins (Mitofusin 1 (*MFN-1*), Mitofusin 2 (*MFN-2*) and Optic Atrophy 1 (*OPA-1*)) [9]. In the fission process, a cytosolic protein called dynamin-related protein (DRP-1) is involved and is responsible for the fission of external mitochondrial membranes [10]. Damaged mitochondria can be enveloped by autophagosomes to trigger their degradation in the lysosome via *PINK-1–PARKIN*-mediated mitophagy pathway proteins [11]. The distribution and movement of mitochondria within the cell is also important for cell function. Mitochondria are transported on cytoskeleton microtubules by molecular motor proteins kinesin and dynein.

MILTON– Mitochondrial Rho GTPase (*MIRO-1*), complex acts as an adapter molecule to link the motor proteins [12].

Despite the high rates of success in embryos obtained after oocyte vitrification, developmental disturbances are seen in some embryos. A number of factors are responsible for these changes. Mitochondrial dysfunctions are also among the changes that occur after freezing, and the mechanism of these changes is not fully known [13]. In our study, we aimed to investigate the expressions of mitochondrial fusion, fission, mitophagy and transport proteins in ovarian tissues by qPCR technique after vitrification.

2. Materials and Methods

2.1 Animals

This study was performed upon the approval by the Animal Experiments Ethical Committee of of Manisa Celal Bayar University Faculty of Medicine. 12 young healthy Balb/c mice were kept at 23 ± 2°C with a humidity of, 55 ± 5% humidity, 12-h light and 12-h dark cycle, and constant ad libitum access to feed food and water.

2.2 Collection of Ovarian Tissue Samples and Application of Vitrification

12 young healthy mice from Manisa Celal Bayar University Experimental Animal Center were induced by intraperitoneal administration of 10 IU of pregnant mare's serum gonadotrophin (PMSG, HOR-272, ProSpec, Israel). 48 hours after induction, ovulation was induced by intraperitoneal administration of 10 IU of human chorionic gonadotropin (hCG, HOR-250, ProSpec, Israel). All animals were euthanized with an overdose of anesthesia (70 mg/kg Ketamine, 10 mg/kg Xylazine) followed by cervical dislocation prior to tissue harvest. The ovaries were removed and examined after

18 hours. The ovaries were placed in disposable petri dishes containing Dulbecco's modified Eagle's medium

(DMEM, Gibco). Each ovary was split into four equal pieces with a sterile surgical blade. The control group was formed by taking 2 parts of each ovarium into a 1.5 mL sterile eppendorf tube. To remove the media, centrifugation was done at 1000 g for 5 min. The supernatant was discarded and 1 mL Ca^{2+} and Mg^{2+} without PBS added into eppendorf tube and washing process was carried out at 1000 g for 5 min. This step was repeated twice.

The ovarian tissues which were used for vitrification were taken into HEPES solution containing 5 mg/mL human serum albumin (HAS, Quinn's, USA). Tissue samples were incubated in equilibration medium [basal medium with 7.5% (v/v) ethylene glycol (EG) and 7.5% (v/v) dimethylsulphoxide (DMSO)] for 5 minutes at room temperature. Then the samples were allowed in vitrification medium [basal medium with 15% (v/v) EG and 15% DMSO] for 60 seconds, placed in polypropylene strips and transferred into the liquid nitrogen. One day after the vitrification, ovary-thawing was performed. The vitrified ovarian tissue samples were taken in warming solution (HEPES-buffered DMEM with 20% FCS) containing 1.0 mol/L sucrose and incubated for 1 min. at room temperature. Ovary tissue samples were then placed in warming solution containing 0.5 mol/L sucrose for 3 minutes at room temperature and washed with the wash solution for 10 min. The samples in the washing solution were centrifuged at 1000 g for 5 min. The supernatant was discarded and 1 mL Ca^{2+} and Mg^{2+} without PBS added into eppendorf tube and washing process was carried out at 1000 g for 5 min. This step was repeated twice.

2.3 Histolopathological Assay

12 ovarian pieces in the control and vitrification groups were fixed in 10% formalin for 24 h, then rinsed under running water overnight. Ovarian tissues were dehydrated through 60, 70, 80, 90% and absolute alcohol, then cleared in xylene followed by saturation in paraffin wax and finally, tissue blocks were prepared with paraffin wax. The 5-µm thick sections were obtained using a rotary microtome (RM 2135, Leica, Germany). Sections were mounted on slides, deparaffinized (overnight at 60°C) and stained with hematoxylin and eosin (H&E). Finally, ovarian morphology was evaluated by light microscopy.

2.4 RNA Isolation from Ovarian Tissues

Ovarian tissues were sliced into small pieces with sterile surgical blade. Total RNA isolation was done by spin coloumn method using TRIZOL and PureLink RNA Mini Kit (Invitrogen, USA) according to manufacturer's introductions with minor modifications. After RNA isolation, RNA quality and concentration were assessed using MaestroNano (MaestroGen, USA) instrument.

2.5 cDNA Synthesis

Complementary DNA (cDNA) was generated by using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). The 20 µl reaction mix contained, 2 µl of 10X RT Buffer, 0,8 µl of 25X dNTP mix, 2 µl of Random Primers, 1 µl of MultiScribeTM Reverse Transcriptase (50U/µl), 5 µl of RNA sample (1 µg) and 9,2 µl of nuclease-free water. Reverse transcription was carried out at 25 °C for 10 min and then at 37 °C for 120 min, followed by an inactivated step at 85 °C for 5 min and a hold step at 4 °C. The cDNAs were stored in -80 ºC until use.

2.6 Primer Design and qPCR

Suitable forward and reverse primers are designed by using Primer 3 software (http://bioinfo.ut.ee/primer3-0.4.0/) for MFN-1, MFN-2, OPA-1, DNM-1, PARKIN, PINK-1, MIRO-1, MILTON and GAPDH genes. The designed primer sets also checked by using NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) software. These primer sets are shown in the Table 1.

Table 1. Sequences of primers for qPCR.

Target Genes	Forward Primer	Reverse Primer
m-MFN-1	5'TTGGCAGGACAAGTAGTGGC3'	5'AGCAGTTGGTTGTGTGACCA3'
m-MFN-2	5'ACTTCTCCTCTGTTCCAGTTGTA3'	5'CAGGGACATCTCGCCAGTTTAT3'
m-OPA-1	5'TTCTGAGGCCCTTCTCTTGT3'	5'TTCTTTGTCTGACACCTTCCTGT3'
m-DNM-1	5'TAGTGGGCAGGGACCTTCTT3'	5'TGCTTCAACTCCATTTTCTTCTCC3'
m-PARKIN	5'GCACACCCAACCTCAGACAA3'	5'GATGACAGAGGAAGATGACTGAC3'
m-PINK-1	5'TGATGTGGAATATCTCGGCAGG3'	5'TGCTTGGGACCATCTCTGGA3'
m-MIRO-1	5'CAATACTGCTGATGCCCCCA3'	5'GTGTCACGTGCGGGTACAT3'
m-MILTON	5'TTGGAGTTTGTGTCTGCCCA3'	5'TGCTGTTGCACACGTCACAT3'
m-GAPDH	5'GGAGAGTGTTTCCTCGTCCC3'	5'ATGAAGGGGTCGTTGATGGC3'

m: mice

qPCR was performed in triplicate for each target and sample by using StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA); a negative control (water) was added for each sample. PCR reactions were carried out in a total volume of 20 µl, consisting of 10 µl of Power Syber Green Master Mix (Applied Biosystems, USA), 2 µl of cDNA (10 ng), 1,5 µl of 500 nm Primer mix (forward+reverse mix for each gene) and nuclease- free water enough to complete the total volume. The qPCR reaction was done at 95 °C for 10 min. (initial denaturation), followed by 40 cycles at 95 °C for 15 s. and 60 °C for 1 min. The relative gene expressions of the target genes were evaluated according to $2^{-\Delta\Delta Ct}$ method. GAPDH gene was used as reference gene to normalize target gene expression levels.

2.7 Statistical Analysis

GraphPad Prism 7.0 (GraphPad Software, California, USA) program was used for statistical analysis. Firstly, the normality test was done to determine whether the value distributions of the groups were normal or not. For this purpose, Shapiro-Wilk test was applied. As the values showed normal distribution, mean values and standard deviations of the groups were determined by student t test. $p<0.05$ was considered statistically significant.

3. Results and Discussion

3.1 Results

Histological evaluation revealed that the control group ovaries showed normal morphology. It was determined that there was no degeneration in both follicles and granulosa cells. Theca cells and the basal membrane were regular (Figure 1A). An abnormal morphology was found in the vitrification group ovaries. It was found that tissue integrity was impaired in some areas, some follicles were degenerated, granulosa cells were shed into the antrum in places and a swab appearance was formed. The theca cells and the basal membrane were normal morphology (Figure 1B).

After total RNA isolation from control and vitrification groups, cDNA synthesis was performed immediately. According to our qPCR results, outer membran fusion proteins *MFN-1* gene expression decreased 1,12±0,08 fold and inner membran protein *OPA-1* increased 1,36±0,15 fold in the vitrification group compared the control group. The mitochondrial fission protein *DNM-1* gene expression increased 1,20±0,03 fold in the vitrification group ct value was not detected. This result suggested that the expression of *MFN-2* gene which is very low in control group is almost completely eliminated after vitrification (Figure 2).

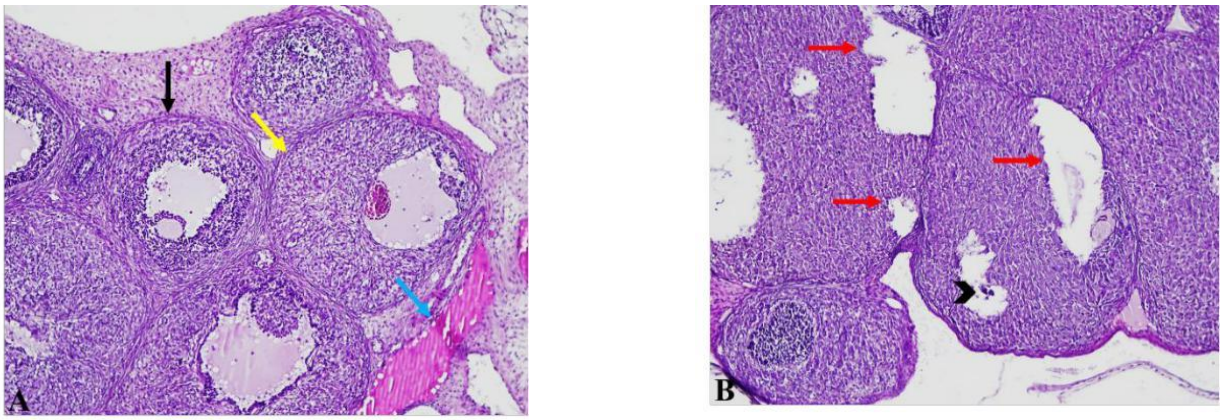

Figure 1. H&E staining of the ovaies. A. The control group ovaries showed normal morphology. The follicles and granulosa cells were intact. B. The vitrification group ovaries were impaired. The dejenerations were observed in some follicles and the granulosa cells were shed into the antrum. Black arrow: secondary follicle, yellow arrow: corpus hemorrhagicum, blue arrow: blood vessel, red arrow: degenerated follicles, black arrowhead: shed granulosa cells. Magnification 100X.

The mitophagy proteins *PINK-1* and *PARKIN* genes expressions decreased 1,34±0,09 and 3,75±0,07 fold respectively in the vitrification group. The transport proteins; *MIRO-1* gene expression decreased 1,16±0,09 fold but *MILTON* gene expression sharply increased 2,28±0,21 compared the control group. *MFN-2* gene Ct value was 34 in control group but in vitrification group ct value was not detected. This result suggested that the expression of *MFN-2* gene which is very low in control group is almost completely eliminated after vitrification (Figure 2).

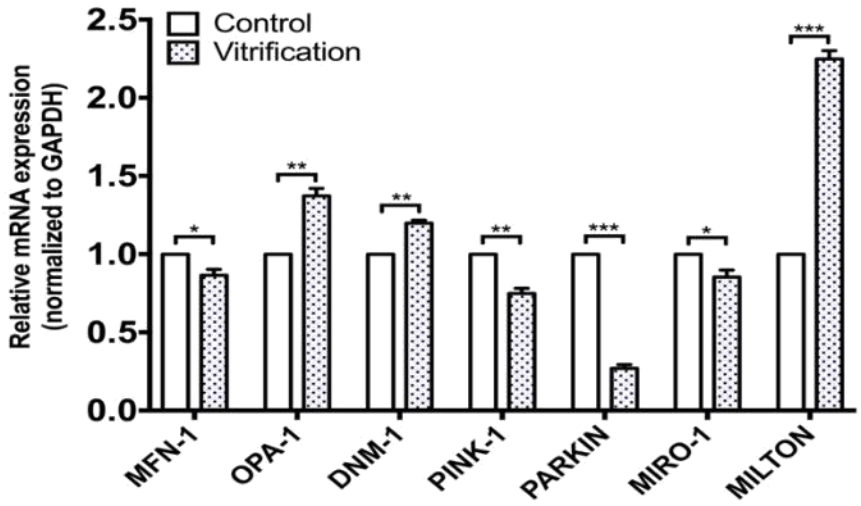

Figure 2. Graphical overview of relative mRNA expressions of target genes. (*p<0,033, **p<0,002, ***p<0,0002)

3.1 Discussion

Vitrification of ovarian tissue is a new alternative method for preserving the fertility of cancer patients before they undergo chemotherapy or radiotherapy [14]. The success of the vitrification of ovarian tissue is limitied because of having many cell types and also developmental stages of oocytes (immature (GV) and MII oocytes). The alteration of the normal mitochondrial function in vitrified/warmed ovarian tissue is reported however the mechanism of mitochondrial disfunction is not known completely [15, 16]. In this study, we investigated the mitochondrial function in mouse ovaries by evaluating the expressions of mitochondrial outer membrane fusion proteins (*MFN*-and *MFN-2*), mitochondrial inner membrane fusion protein (*OPA-1*), fission protein (*DNM-1*), mitophagy proteins (*PARKIN* and *PINK-1*) and transport proteins (*MIRO-1* and *MILTON*) with qPCR technique.

Vitrification is a rapid freezing technique and is claimed to make minimal changes in ovarian tissue. However, vitrification has been reported to cause cell membrane defects, cytoskeleton damage, mitochondrial depolarization and increased reactive oxygen species. It is known that mitochondria are affected during vitrification. The mitochondrial distribution in MII oocytes and embryos is impaired in Mouse [17] and the ATP concentration dropped significantly in humans [6]. Similar results have been demonstrated in the slow freezing technique in human MII oocytes [18]. When the distribution of mitochondria in the oocyte was examined, mitochondria are accumulated in the regions where energy is needed. However, the distribution of mitochondria within the frozen oocyte is different from fresh oocyte. It is suggested that this alteration is due to microtubule-mediated mitochondrial dysplasia [19]. Knowledge on mitochondrial damage after ovarian freezing is controversial. It has been suggested that ovarian tissue vitrification did not alter mitochondrial distribution in mouse GV oocytes but the internal membrane potential changed and normalized after 12 days of culture [5]. Mojdeh Salehnia et al. suggest that the ATP level decreases in the frozen group, while the mitochondrial inner membrane potential does not change [16].

MFN-1 acts as a mediator that provides mitochondrial fusion and hemostasis. In *MFN-1* null mouse, mitochondrial fusion decreased and numerous mitochondrial fragmentations were observed. When generating knockout mouse for *MFN-1*, failure of mitochondrial fusion causes decreased mitochondrial membrane potential and oxidative phosphorylation. This leads to a decrease in ATP concentration [20]. In our study, we have found the *MFN-1* gene expression decreased 1,12±0,08 fold in the vitrification group compared the control group and this decrease of *MFN-1* expression in ovarian tissue may affect the oocyte maturation and embryo development. The immature and mature mouse oocytes express the *MFN-1* proteins at similar levels and mitochondrial aggregation was particularly concentrated around the chromosomes during maturation of these oocytes [21]. Hua S et al.

showed that high level of *MFN-1* expression significantly improved the embryo development rates by increasing ATP level and Δψm, while reducing H2O2 generation [22]. *MFN-2* is a mitochondrial outer membrane protein and is involved in the mitochondrial fusion function [9]. In our study, we observed that MFN2 gene Ct value was 34 in control group, but ct value of the *MFN-2* gene in vitrification group was not detected. According to this result, we thought that the expression of *MFN-2* gene which is very low in control group is almost completely eliminated after vitrification and may related to concominant alterations in mitochondria function. Dai J. et al. showed that the expression of *MFN-2* gene was slightly decreased in the porcine MII oocytes and suggested that vitrification had an adverse effect on mitochondria [23]. In additon this, *MFN-2* is essential for embryonic development, and a dramatic disruption in placental development, most obviously in the paucity of trophoblast giant cells were seen in *MFN-2*-deficient mice [24].

Our qPCR results have shown that *OPA-1* gene expression increased 1,36±0,15 fold in vitrification group. We have thought that this increased *OPA-1* expression in the vitrified ovarian tissue is due to protect the cells from apoptosis after cell damage. *OPA-1* is a necessary protein for inner membrane fusion of mitochondria. Lack of *OPA-1* causes mitochondrial fusion disorder, crista membrane disorganization. *OPA-1* might be a key protein that modulates the inner membrane dynamic to either maintain cell homeostasis or commit them to apoptosis and consequently could be a target for pro- or anti-apoptotic effectors [25]. Boucret L et al. reported that the *OPA-1* expressed by cumulus cells and may play a major role in the constitution of a sufficient mtDNA pool, essential to oocyte competence, and that this role may be impaired in patients with diminished ovarian reserve [26]. Conca CD et al. investigated the mouse model of fragile X primary ovarian insufficiency, the *OPA-1* expression in whole ovaries was significantly lower in premutation mice when compared the wild type mouse and claimed that knockdown of *OPA-1* resulted in smaller mitochondria with an 'empty' appearance as seen in the mice [27].

The *DNM-1* protein is involved in the mitochondrial fission and we have found that *DNM-1* expression increased 1,2±0,03 fold in the vitrification group compared the control group. The increase of the *DNM-1* expression may be related with the mitochondrial damage. Dai J et al. have shown that *DNM-1* is present in the fresh porcine MII-stage oocytes and of *DNM-1* expression upregulated in vitrified oocytes. They claimed that the *DNM-1* gene expression of vitrified oocytes could reflect the injury of mitochondrial function. The damage to mitochondria after vitrification could stimulate the intrinsic apoptotic pathway and induce the occurrence of apoptosis [23].

In mammalian cells, specific mitophagy programs are activated to remove mitochondria during erythrocyte maturation, destroy sperm-derived mitochondria after oocyte fertilization and eliminate damaged mitochondria

[28]. Mitochondria-specific autophagy, mitophagy, is a key mitochondrial quality control mechanism that helps maintain mitochondrial fitness by efficiently removing dysfunctional organelles. Although several different mechanisms regulating mitophagy have been described in mammalian cells, the best understood is PTEN-induced putative kinase 1 (*PINK-1*)/*PARKIN*-mediated mitophagy [29]. In our study, we have found that *PINK-1* and *PARKIN* genes expressions decreased 1,34±0,09 and 3,75±0,07 fold respectively in the vitrification group.

Depletion of *PINK-1* causes moderate fragmentation of functional mitochondria in mammalian cells, and a slight accumulation of depolarized mitochondria. *PINK-1*-deficient cells display increased levels of reactive oxygen species, impairment of stimulated mitochondrial Ca^{2+} uptake and decreased mitochondrial ATP synthesis [30]. Recent evidences also show that *PARKIN* loss leads to a marked decrease in mitochondrial biogenesis, mitochondrial dysfunction and enhanced levels of reactive oxygen species [31].

MILTON protein acts as an adapter molecule in the mitochondrial transport. *MIRO-1* protein locates on the outer mitochondrial membrane and binds to the *MILTON* [12]. Although the main function of the *MILTON* in cells is mitochondrial transport, it also plays an important role in the mitochondrial fusion function. Fusion does not occur in the cells without *MILTON*. It is known that excess of *MILTON* causes enlarged mitochondria [32]. Mitochondria migrate to the region inside the cells where energy is needed. It is reported that ADP/ATP ratio is important in mitochondrial movements and increased ADP concentration inhibits motor functions in the cells. Also, cytoplasmic calcium ion concentration is important in this process. It has been reported that the movement decreases to the region with high Ca^{2+} concentration [33]. Reactive oxygen species are produced and eliminated in mitochondria. Therefore, it's suggested that ROS affects mitochondrial shape and distribution [34]. Increased ROS production and oxidative stress has been reported to occur after ovary freezing [35]. Cao et al. have determined the differences in mitochondrial distribution in the fox oocytes after vitrification [36]. According to qPCR results in our study, we have found that *MIRO-1* expression decreased 1,16±0,09 fold and *MILTON* expression increased 2,28±0,21 fold in the vitrification group. This increase in the expression of the *MILTON* was thought to be related to the increase in mitochondrial transport and ROS production.

4. Conclusion

Histological evaluation revealed approximately similar integrity in the vitrified ovaries compared to the control. Some spaces were seen between oocyte-granulosa cells and surrounding granulosa cells, and oocyte shrinkage

was also observed in the vitrification group. We observed changes in the expression of the mitochondrial fusion (*MFN-1*, *MFN-2* and *OPA-1*), fission (*DNM-1*), mitophagy (*PARKIN*, *PINK-1*) and transport (*MIRO-1*, *MILTON*) proteins. Our results suggest that vitirification effects the mitochondrial gene expressions. The alternation of the gene expressions may concomitant with a decrease in the mitochondrial function during the ovarian vitrification and may effects the oocyte maturation and embryo development.

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