IJMS
Vol 44, No 5, September 2019

Original Article

Overexpression of Mitochondrial Genes (Mitochondrial Transcription Factor A and Cytochrome c Oxidase Subunit 1) in Mouse Metaphase II Oocytes following Vitrification via Cryotop

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Received: 1 January 2018
Revised: 10 February 2018
Accepted: 25 February 2018

What's Known

- Vitrification is the main approach to human oocyte and embryo cryopreservation and can be considered for increasing IVF cycle efficiency. Oocyte viability levels after vitrification are dependent on the concentrations of cryoprotectant agents (CPAs), exposure time to CPAs, temperature of equilibration solutions, vitrification speed, and cell size.

What's New

- We sought to determine the effects of vitrification on the expression levels of mitochondrial genes, including mitochondrial transcription factor A (Tfam) and cytochrome c oxidase subunit 1 (Cox1). Currently, there are inadequate data on the effects of freezing/thawing on the cytoplasmic maturation of oocytes and regulation of mitochondrial transcription genes.

Abstract

Background: Gamete cryopreservation is an inseparable part of assisted reproductive technology, and vitrification is an effective approach to the cryopreservation of oocytes. The aim of this study was to investigate vitrification effects on the expression levels of mitochondrial transcription factor A (Tfam) and mitochondrial-encoded cytochrome c oxidase subunit 1 (Cox1) in mouse metaphase II oocytes.

Methods: Oocytes were selected by simple random sampling and distributed amongst five experimental groups (control [n=126], docetaxel [n=132], docetaxel+cryoprotectant agent [CPA] [n=134], docetaxel+vitrification [n=132], and vitrification [n=123]). After the warming process, the oocytes were fertilized and cultured into a 2-cell stage. Then, the effects of vitrification on the expression of the Tfam and Cox1 genes were determined via real-time reverse transcriptase polymerase chain reaction. Each group was compared with the control group. The data were analyzed with ANOVA using GraphPad and SPSS, version 21.

Results: A significant decrease was observed in the fertilization rate of each group in comparison with the control group (P=0.001). The rate of 2-cell formation after in vitro fertilization was significantly lower in both vitrification groups (docetaxel+vitrification and vitrification) than in the non-vitrification groups (fresh control and docetaxel) and control group (P=0.001 and P=0.004). The expression level of Cox1 was significantly higher in the vitrification group than in the control group (P=0.01), while it was lower in the docetaxel group than that in the control group (P=0.04). The expression level of the Tfam gene was significantly high in the vitrification group (vitrification+docetaxel) and the non-vitrified group (docetaxel+CPA) in comparison with the control group (P=0.01).

Conclusion: This study indicated that the vitrification of mouse MII oocytes increased the expression of the Tfam and Cox1 genes.

Please cite this article as: Dehghani N, Dianatpour M, Hosseini SE, Khodabandeh Z, Daneshpazhouh H. Overexpression of Mitochondrial Genes (Mitochondrial Transcription Factor A and Cytochrome c Oxidase Subunit 1) in Mouse Metaphase II Oocytes following Vitrification via Cryotop. Iran J Med Sci. 2019;44(5):406-414. doi: 10.30476/IJMS.2019.44960.

Keywords ● Vitrification ● Oocytes ● Docetaxel ● Mitochondrial transcription factor A

Introduction

Gamete cryopreservation is an integral part of assisted reproductive technology and includes in vitro fertilization (IVF),

which is wildly used in clinical practice. Moreover, cryopreservation is an established method to preserve fertility in women who are at risk of cancer and autoimmune diseases.[1] The cryopreservation of oocytes and embryos is an opportunity to increase the efficiency of IVF cycles in several species, including mice,[2] rabbits,[3] bovines,[4] and humans.[5] Vitrification is the main approach to the cryopreservation of human oocytes and embryos. This method is a physical process through which a high concentrated cryoprotectant agent (CPA) prevents the formation of ice crystals.[6, 7] It is the exceptional advantage of vitrification among the cryopreservation methods.

A variety of factors during vitrification might affect oocytes, including the toxicity of highly concentrated CPAs, cold shock, and osmotic stress. These factors may destroy microfilaments and organelles, harden zonae pellucidae, and damage chromosomes.[8] The level of oocyte viability after vitrification is dependent on the CPA concentration, time of exposure to the CPA, temperature of the equilibration solution, speed of vitrification (all steps), and cell size. Since mammalian oocytes are large in size, they are more prone to damage by changes in the temperature and concentration of CPAs.[9-11] Therefore, the results can be improved by changing the vitrification condition.

Spindle fiber stabilization plays a critical role in the successful vitrification of oocytes. Docetaxel, an anti-cancer drug, inhibits the depolymerization of microtubules, which stabilizes them by binding them with the b-subunit of tubulin in microtubules. Thus, damage to cytoskeleton fibers can be reduced by docetaxel during vitrification, and the viability of oocytes might improve after vitrification/warming.[3, 12]

There are many different types of permeable cryoprotectants used for vitrification, including ethylene glycol (EG), 1,2-propanediol, and dimethyl sulfoxide (DMSO). Sucrose is the most common non-permeable CPAs and is used in oocyte vitrification. A previous study reported that a mixture of DMSO and EG in identical quantities was the most efficient method.[13] EG has a low molecular weight, high permeation ability, and low toxicity, making it suitable for use in the vitrification of human oocytes and embryos. Furthermore, EG permeability can be facilitated by DMSO.[14] High concentrations of CPAs might induce osmotic stress; consequently, establishing stability between high concentrations of CPAs and low toxicity can reduce this negative effect.[15]

Mitochondria have their own genetic material, known as mitochondrial DNA (mtDNA). In other words, the function of mitochondria is interconnected with mtDNA.[16] The cytoplasmic maturation and developmental competence of oocytes are related to mitochondrial distribution. A previous study showed the direct effect of gene-specific transcription factors on gene transcription in mitochondria.[17] Mitochondrial transcription factor A (Tfam) regulates mtDNA transcription and replication in several tissues. Tfam, a nuclear-encoded high-mobility group box protein, acts as the regulator of mtDNA promoters.[18] Cytochrome c oxidase subunit 1 (Cox1) is one of the three genomic components of the mitochondrial respiratory chain.[19] Novin and others[20] showed that Cox1 could be considered an indirect indicator of mtDNA activity and quantity. There are currently inadequate data on the effects of freezing/thawing on the cytoplasmic maturation of oocytes and the expression regulation of mitochondrial genes.

To the best of our knowledge, gene expression has not been completely investigated in mammalian vitrified oocytes. Hence, the objective of the present study was to determine the expression of some mitochondrial genes, including Tfam and Cox1. Additionally, the effects of oocyte freezing via the Cryotop method with the mixture of CPAs, as well as the effects of docetaxel on the survival, morphology, and gene expression of mouse oocytes, were examined.

Materials and Methods

The present experimental study was conducted using mouse oocytes and sperm. All the trials on the animals were approved by the Ethics Committee of Islamic Azad University based on the rules for working with laboratory animals of Iran Veterinary Organization (ethical code: IR.MIAU.REC.1397.801). Female (8–10 weeks old) and male (10–12 weeks old) Naval Medical Research Institute (NMRI strain) mice were purchased from Royan Institute (Karaj, Iran). The animals were kept in a 12-hour light/dark cycle at a controlled temperature (22±2 °C) and humidity (40%–50%) for 2 weeks to adapt to laboratory conditions. Standard rodent chow and water were available *ad libitum*.[21, 22]

Oocyte Collection

Superovulation was done with intraperitoneal injections (i.p.) of 10 IU of pregnant mare's serum gonadotropin (Sigma, UK), followed by 10 IU of human chronic gonadotropin 48 hours later.[23] All the female mice were sacrificed by cervical dislocation between 13 and 15 hours following the administration of human chronic gonadotropin. The cumulus complexes of the oocytes were isolated from the antral follicles

and placed in a base medium (G-MOPS™), containing 300 µg/mL of hyaluronidase (Code 4272, Sigma, UK) for 30 seconds to 1 minute.[23] Typical MII-stage oocytes were selected based on the presence of a first polar body and having homogenous ooplasm under a light microscope (Olympus, Tokyo, Japan) for the experimental groups. The ones with dark ooplasm were considered dead.

Experimental Design

There were five treatment groups: group I: untreated oocytes (fresh control); group II: oocytes preincubated with docetaxel for 20 minutes (docetaxel); group III: oocytes preincubated with docetaxel for 20 minutes and then exposed to CPAs, but not vitrified (docetaxel+CPA); group IV: oocytes preincubated with docetaxel for 20 minutes and vitrified through the Cryotop method (docetaxel+vitrification); and group V: oocytes vitrified via the Cryotop method (vitrification).

All the oocytes (vitrified oocytes after the warming process) were evaluated for morphology and survival. The oocytes were analyzed using real-time polymerase chain reaction (PCR) to evaluate the expression level of Tfam and Cox1 in all the groups.

Preincubation of the Oocytes with Docetaxel

The oocytes in each group, except for those in the control and vitrification groups, were preincubated with docetaxel (Sigma-Aldrich, UK) at a concentration of 0.05 µM for 20 minutes.[24]

Vitrification and Warming

G-MOPS™ (Vitrolife, Sweden AB, Göteborg, Sweden) was used as a carrier for the cryoprotectants. Denuded MII oocytes were vitrified in groups of 15, using the Cryotop as a device.[5]

The CPAs were supplemented with 15% EG (Sigma-Aldrich, Steinheim, Germany) plus 15% DMSO (Sigma-Aldrich) plus 0.5 M of sucrose (Sigma-Aldrich, UK) in the base medium. The equilibration solutions were half of the CPAs with no sucrose. Briefly, 15 oocytes were incubated in the base medium (G-MOPS™), containing 0.05 µM of docetaxel for 20 minutes. The oocytes were equilibrated in the first equilibration drop for 3 minutes. Then, they were transferred to the vitrification solution for 1 minute. Finally, the oocytes were rapidly placed on top of the cryotops (Kitzato Ltd., Tokyo, Japan). The excess media around the oocytes were precisely removed, and the cryotops were promptly plunged into liquid nitrogen (LN2).[11]

For the warming process, the cryotops were directly inserted into a thawing solution (1M of sucrose, G-MOPS™) for 1 minute at 37˚C. Next, the oocytes were placed into reduced sucrose concentrations (0.5 M for 3 min and 0.25 M for 5 min) and the medium was kept for 3 to 5 minutes.

All the vitrified/warmed oocytes were visualized under a light microscope to assess their viability in terms of normal-appearing zonae pellucidae, integral plasma membranes, and intact polar bodies. The vitrification process was done at room temperature (25 ˚C) according to the protocol described in our previous studies.[6, 25]

Sperm Preparation

The male mice (8–12 weeks old) were sacrificed by cervical dislocation, and their cauda epididymis was excised and chopped. The sperms were capacitated in a medium (HAM's F10 [Sigma]+4 mg/mL of bovine serum albumin [Sigma-Aldrich]) at 37°C in 5% CO_2 between 45 and 60 minutes.

IVF

The vitrified/warmed oocytes with intact zonae pellucidae, intact plasma membranes, and homogeneous cytoplasms were chosen and placed in 200 µL drops of a G-IVF medium (Vitrolife, Sweden) layered under mineral oil (Sigma, 8410). The medium had been prepared earlier and incubated at 37 °C in 5% CO_2 for 2 hours. A final concentration of 2×10^6 spermatozoa/mL was added to the G-IVF medium containing 15 oocytes and incubated at 37 °C in 5% CO_2 for 6 hours. The percentages of the formation of 2 pronuclei were recorded to evaluate the fertilization rate.

The oocytes were thereafter placed in 25 µL drops of a cleavage medium (G1, Vitrolife, Sweden) and incubated at 37 °C in 5% CO_2. The rate of 2-cell formation was evaluated 24 hours later under an inverted microscope (Nikon, Japan).

RNA Extraction and Real-Time PCR

Total RNA was isolated from the vitrified and non-vitrified oocytes, using an RNA extraction kit (CinnaGen Inc., Iran). The quantity and quality of RNA were checked by evaluating the optical density of 260/280 nm (NanoDrop™ spectrophotometer) and agarose gel (1%) electrophoresis. Totally, 1000 ng RNA was used to synthesize the first-strand complementary DNA (cDNA) using a RevertAid™ First Strand cDNA Synthesis kit (Fermentas Inc.). Quantitative real-time polymerase chain reaction (qPCR) was performed using the Applied Biosystems StepOne and the Real Plus 2x Master Mix Green (Amplicon Inc.). The primers were designed based on mouse DNA sequences, found in the gene bank Primer-BLAST online program

Table 1: Sequences of the primers used for real-time polymerase chain reaction

Gene	Primer Sequence(5'-3')	Size (bp)
Tfam	F:CACCCAGATGCAAAACTTTCAG R:CTGCTCTTTATACTTGCTCACAG	147
Cox1	F:GATTGTACTCGCACGGGCTAC R:GGATAAGGTTGGACCGCACT	202
β-actin	F:AGTGTGACGTTGACATCCGT R:TGCTAGGAGCCAGAGCAGTA	120

Tfam: Mitochondrial transcription factor A; Cox1: Cytochrome c oxidase subunit 1

(table 1).[26] The β-actin gene was also used as the internal control of the qPCR reactions. The qPCR conditions were set at 10 minutes at 94 °C, followed by 40 cycles of 15 seconds at 94 °C, 60 seconds at 58 °C, and final extension of 7 minutes at 72 °C. The amplification signals of the different samples were normalized to the cycle threshold of β-actin. Subsequently, the delta-delta cycle threshold ($2^{-\Delta\Delta CT}$) method was applied to compare the mRNA levels between the activated and the control groups, which represented as fold change in the data analyses. All the experiments of the real-time reverse transcriptase polymerase chain reaction (RT-PCR) were triplicated.

Statistical Analysis

The statistical analyses were carried out using SPSS, version 21. One-way analysis of variance (ANOVA) and the post hoc Tukey test were used to determine the differences between the mean values of the survival and fertilization of the oocytes. The expression levels of the genes were analyzed using GraphPad Prism. The normality and homogeneity of the data were tested before the analyses.

Results

Oocyte Viability and Fertility After Vitrification

The survival rate of each group was separately compared with that of the control group. As is shown in table 2, the survival rate of the vitrified/warmed oocytes was higher in the docetaxel+vitrification group than in the vitrification group (P=0.947), but the difference was not significant. Similarly, there was no significant difference in oocyte survival between the docetaxel group and the docetaxel+CPA group (P=0.533). The survival rate of the vitrification group was, nevertheless, significantly lower than that of the control group (P=0.033).

The results showed a significant decrease in the fertilization rate of each group in comparison with the control group (P<0.001). The rate of 2-cell formation after IVF was significantly lower in both vitrification groups (docetaxel+vitrification and vitrification) than in the non-vitrification groups (fresh control and docetaxel) (table 2).

Quantitative Real-Time RT-PCR

The expressions of the Cox1 and Tfam mRNA were evaluated in the vitrified/warmed and non-vitrified groups and then compared with those of the control group. According to the results (figure 1), the expression level of the Cox1 gene in the vitrification group was significantly higher than that of the control group (P=0.01), whereas the expression level of the Cox1 gene in the docetaxel group was significantly lower than that in the control group (P=0.04).

Table 2: Effects of vitrification on the survival and the fertilization rate of the MII oocytes

Variable	Survival of the Oocytes after Vitrification mean±SEM	P value	Oocyte Fertilization (2-cell formation) mean±SEM	P value
Fresh control (group I)	124/126 (98.4±1.5) [a]	a=0.033	80/71 (88.6±3.2) [a]	a=0.001
Docetaxel (group II)	122/132 (92.6±3.2)		99/83 (82.8±5.2) [b,c,d]	b=0.001 c=0.030 d=0.004
Docetaxel+CPA (group III)	113/134 (84.1±3.3)		105/80 (76.95±2.03)[a,b]	a=0.001 b=0.001
Docetaxel+vitrification (group IV)	111/132 (85±3.8)		115/75 (65.3±3.2)[a,b]	a=0.003 b=0.030
Vitrification (group V)	98/123 (81.1±5.8)[a]		105/61 (60.4±4.6)[a,b]	

CPA: Cryoprotectant agent; Tukey method was used for multiple comparisons. Data are shown as mean (%)±SEM of three replications. Two-cell formation was observed at 24 hours after in vitro fertilization. a: Indicates significant differences between group I and groups II, III, IV, and V; b: Indicates significant differences between group II and groups III, IV, and V; c: Indicates significant differences between group III and groups IV and V; d: Indicates significant differences between group IV and group V

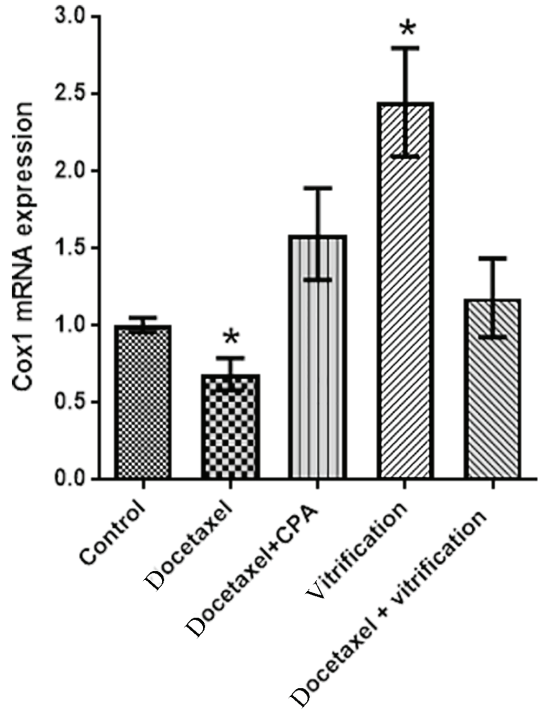

Figure 1: The expression levels of cytochrome c oxidase subunit 1 (Cox1) are shown in different groups of vitrified and non-vitrified MII oocytes. *Significant differences between the docetaxel and vitrification groups and the control group (P=0.049 and P=0.015, respectively)

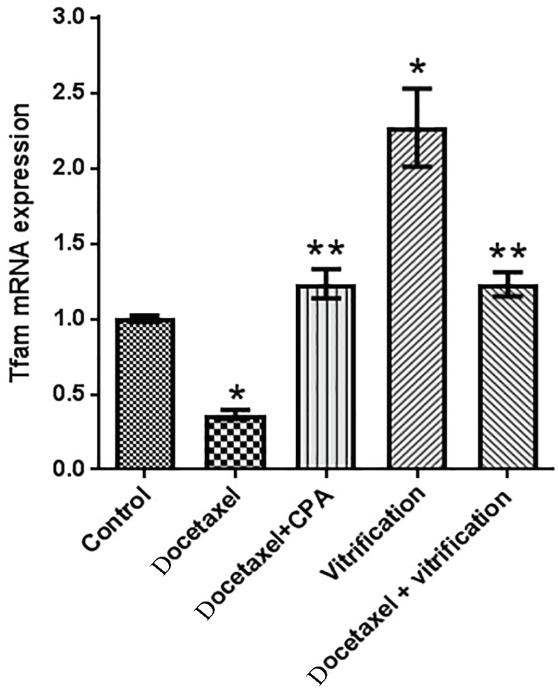

Figure 2: The expression levels of mitochondrial transcription factor A (Tfam) are shown in different groups of vitrified (control, docetaxel, and docetaxel+cryoprotectant agent [CPA]) and non-vitrified (vitrification and docetaxel+vitrification) MII oocytes. *Significant differences between the docetaxel and vitrification groups and the control group (P=0.001 and P=0.001, respectively); **Significant differences between the docetaxel+cryoprotectant agent (CPA) group and the docetaxel+vitrification group and the control group (P=0.023 and P=012, respectively)

The results showed that the expression level of the Tfam gene was significantly higher in the vitrification group (P=0.001) (figure 2). The expression level of the Tfam gene was significantly higher in the docetaxel group (P=0.001), the docetaxel+CPA group (P=0.023), and the docetaxel+vitrification group (P=0.012) than in the control group.

Discussion

We observed that the Cryotop changed the transcription levels of mitochondrial genes, including Tfam and Cox1, in the vitrified/warmed MII oocytes.

The present study showed that the pretreatment of oocytes with docetaxel before vitrification changed the transcript level of Tfam and Cox1, while some other conditions failed to do so. Mitochondria are considered critical organelles in oocytes. Mitochondria are completely transferred to zygotes from oocytes; hence, the fertilizability of the mitochondrial genome of oocytes is very important.[27] Novin and colleagues[20] showed that a reduction in mitochondrial activity led to damage to oocyte maturation and caused a defect in embryo development. The authors, therefore, concluded that there was a relationship between oocyte maturation and the overexpression of mitochondrial-related (NRF1, Tfam, and MT-CO1) genes in human oocytes.

Bartolac and others[28] reported that after the warming process, the survival rates of their closed and open vitrification systems were similar. Therefore, the differences in the gene expression levels between various groups were not related to post-warming survival.

Docetaxel stabilizes microtubules by inhibiting the rate and extent of tubulin assembly during vitrification.[29] In the current study, the survival and fertilization rates of the vitrified/warmed oocytes that were preincubated with docetaxel were better than those of the oocytes without preincubation. It has been suggested that the normality of cytoskeleton fibers, cortical granules, and mitochondria after vitrification/warming might be associated with the level of cell metabolism, proliferation, and differentiation. One of the main cryo-injuries in vitrified/warmed oocytes is damage to the cytoskeleton fiber system of oocytes. Normal fertilization is closely related to normal cortical distributions in MII oocytes.[24] Chasombat and colleagues[24] showed

that the preincubation of in vitro-matured bovine oocytes with docetaxel had no toxic effect on the survival and fertilization rate of the vitrified/warmed oocytes.

After the processes of vitrification and warming, meiotic spindles are recovered and the development of the embryo is improved. Thus, vitrified/warmed oocytes are comparable in cleavage timing, cell number, and DNA methylation patterns with fresh oocytes.[30, 31]

We observed that mitochondrial gene expression was increased in the vitrified/warmed and non-vitrified groups compared to the control group. Contrary to our expectations, however, the increase in the mRNA expression level of the Tfam and Cox1 genes might have been a result of high mitochondrial biogenesis and the stimulation of mitochondrial respiratory activity. Moreover, there is a direct relationship between the cytoplasmic microtubule arrangement and the normal distribution of active mitochondria, which leads to normal ATP circulation in the cell. This can be achieved by using docetaxel before vitrification to stabilize microtubules.[16] Our findings showed that the pretreatment of the oocytes with docetaxel before vitrification, as well as the vitrification method, was able to increase mitochondrial gene activity. Docetaxel can also affect mitochondrial permeability membrane, which might activate caspases.[32] That is what might have led to the significant decrease in the level of mitochondrial genes in the docetaxel group compared with the control group in the present study. The failure of mitochondrial activity might cause the potential deterioration and disruption of the membrane. The potential deterioration in the membrane is reversible, while membrane disruption is deemed a serious damage. A recent study showed that swollen mitochondria were detected more frequently after vitrification, but no rupture of mitochondrial membrane was observed. This shows that some ultrastructural changes in embryos and oocytes after vitrification are reversible, and they can fully recover.[33] We observed that the expression levels of the mitochondrial genes (Tfam and Cox1) in the vitrification group were significantly increased by comparison with the control group, which was an indication of the full recovery of the oocytes after vitrification. These genes are also associated with RNA metabolism and are very important in oocyte maturation. Oocytes need to accumulate a large number of RNA to preserve fertilization, early embryonic development, and embryonic genome activation. The high gene expression can be related to RNA metabolism, which is critical during oocyte maturation and development.[20]

The Tfam gene has an important role in regulating the copy number of mtDNA; hence, oocyte developmental stages are associated with the transcript levels of this gene. The data from a previous study showed a reduction in Tfam expression in the vitrified oocytes in comparison with the non-vitrified group. This result might be due to the critical role of Tfam in the stability of mtDNA.[34] There are 1000 Tfam molecules per one mtDNA in each nucleoid. Kukat and others[35] showed that the Tfam gene activated the mitochondrial biogenesis program and played a critical role in the biogenesis and stability of mtDNA and the regulation of the oxidative phosphorylation of cells during development and adaptation. The authors concluded that mtDNA transcription was an important step in the biogenesis of the whole organelle, especially in mitochondria. Consequently, a low expression level of the Tfam gene might be due to reduced mtDNA copy numbers.[34]

Cox1 is one of the three mtDNA-encoded subunits of cytochrome c oxidase and is considered a critical enzyme in the respiratory chain. The Cox1 transcript level indirectly shows the activity and quantity of mtDNA. Furthermore, the activity and proficiency of the mitochondrial transcription apparatus might be measured by the relative abundance of mtDNA products.[36] Cox1 plays a vital role in oocyte developmental competence. May and others[37] reported that the bovine oocytes with fewer transcripts of Cox1 failed to cleave after IVF. The data in our study showed that the vitrified/warmed oocytes with the Cryotop method increased mitochondrial gene expression. Nile and colleagues[38] proved that there was a direct relationship between partial mtDNA reduction and mitochondrial function. The investigators also showed a significant decrease in basal and maximal respiratory capacity, as well as a significant decrease in ATP production by oxidative phosphorylation in the Tfam-silenced cells compared with the control cells.

Manczak and colleagues[39] reported that the upregulation of mitochondrial genes might be a compensatory response due to the generation of free radicals during aging. Some genes might be more disposed to damage (oxidative and stress), whereas others might compensate to control the damage.

The upregulation of these mitochondrial genes during vitrification, as well as aging, might be because mitochondria are very sensitive. In other words, mitochondria try to compensate for the loss of their function and oxidative damage, which may occur in the electron transport chain.

Conclusion

The results of the present study showed that docetaxel pretreatment of mouse MII oocytes before vitrification increased mitochondrial gene expression. Thus, high transcript levels of mitochondrial genes (Tfam and Cox1) lead to an improvement in the quality and developmental competence of oocytes after vitrification in assisted reproductive technology.

Acknowledgment

The results presented in this paper were part of a student thesis (thesis number: 16330525941001 and acceptance date: 02.07.2017). This study was financially supported by the Stem Cells Technology Research Center, Shiraz University of Medical Sciences, and the Department of Biology, College of Science, Fars Science and Research Branch, Islamic Azad University, Shiraz, Iran. The authors would like to thank Mr. H. Argasi at the Research Consultation Center (RCC) of Shiraz University of Medical Sciences for his invaluable assistance in editing this article.

Conflict of Interest: None declared.

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