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Original Article

Protective Effect of Docetaxel Against Autophagy-Related Genes in Vitrification of Mouse Metaphase II Oocytes

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Abstract

Background: Autophagy is a conservative mechanism for cell survival as the main response of cells to stress conditions. The present study aimed to assess the effect of docetaxel on the survival, fertilization, and expression of autophagy-related genes in vitrified oocytes.

Methods: The study was conducted in 2018 at the Stem Cells Technology Research Center, Shiraz University of Medical Sciences (Shiraz, Iran). Denuded oocytes were randomly selected and assigned to five groups, namely control (n=133), docetaxel (n=136), docetaxel+cryoprotectants (n=146), docetaxel+vitrification (n=138), and vitrification (n=145). The effect of vitrification on the expression of autophagy-related gene 5 (*ATG5*) *and Beclin-1* was determined using a real-time polymerase chain reaction. Data were analyzed using SPSS software (version 26.0) and GraphPad Prism 9.

Results: Survival and fertilization rates in each experimental group were significantly reduced compared to the control group (P=0.001). After *in vitro* fertilization of oocytes, the 2-cell formation rate was significantly reduced in the docetaxel+vitrification and vitrification groups compared to the control and docetaxel groups (P=0.001 and P=0.001, respectively). Pre-incubation of oocytes with docetaxel reduced gene expression levels of *Beclin-1* and *ATG5* in the docetaxel+cryoprotectants and docetaxel+vitrification groups (P=0.001 and P=0.019, respectively). The expression level of these genes was also reduced in the docetaxel group compared to the control group (P=0.001).

Conclusion: Incubation of mouse metaphase II oocytes with docetaxel prior to vitrification reduced the expression of autophagy-related genes and increased survival and fertilization rates compared to untreated oocytes.

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Keywords ● Oocytes ● Vitrification ● Docetaxel ● Autophagy ● *Beclin-1* ● *Atg5*

What's Known

- Vitrification is a fast-freezing technique to preserve human oocytes. High concentrations of cryoprotectants are used to prevent ice crystal formation. Vitrification quality depends on cryoprotectant concentration and cell exposure time.
- To date, there are limited studies on the effect of vitrification on oocyte maturation and transcriptional regulation of autophagy.

What's New

- Incubation of mouse metaphase II oocytes with docetaxel before vitrification reduced the expression of autophagy-related genes.
- Pre-incubation with docetaxel significantly improved the viability and developmental competence of oocytes.

Introduction

Autophagy is a highly conserved cellular recycling process, which plays an important role in maintaining cellular homeostasis by recycling long-lived proteins and damaged organelles.[1, 2] This process involves the removal of subcellular structures,

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such as redundant peroxisomes and damaged mitochondria and endoplasmic reticulum.[3] Conserved energy through the autophagy of intracellular protein and organelles allows the body to adapt to different conditions. Some studies showed the association of autophagy with physiological and pathological processes of the body, such as cell growth, differentiation, and development; immune responses, cancer, neurodegeneration, and metabolic diseases.[4, 5] It is also reported that autophagy plays a role in aging, illnesses, antigen presentation, and bacterial infections.[6] Moreover, it affects fertilization by degrading cytoplasmic components in the lysosome. A previous study on mouse embryos reported that after fertilization, degradation of maternal proteins in oocytes occurred and new proteins were synthesized by the zygotic genome. Breakdown of autophagy-related gene 5 (Atg5), as maternal protein, is compromised in the Atg5-null sperm and affects the normal translational regulation and possibly the transcription regulation.[7] In addition to *Atg5*, there are several other autophagy-related genes, such as *Atg6*/*Beclin-1*, *Atg8*/LC3, *GABARAP*, *Atg4A*, and *Atg4B*. These genes are involved in the molecular mechanism of autophagy and have been studied in various cells.[8]

Atg5 and *Beclin1*, the mammalian orthologue of yeast *Atg6*, are critical factors in autolysosome formation that induces autophagy.[9] In the absence of *Atg5* and *Atg7*, essential for the formation of autophagosomes, oocytes are normally fertilized *in vivo*.[10] Tsukamoto and colleagues showed that when autophagy-deficient oocytes (derived from *Atg5* knockout mice) are fertilized with *Atg5*-null sperm, they only develop up to the 4- and 8-cell stages. However, they can fully develop if fertilized by wild-type sperm.[7] *Beclin-1*, a homolog of *Atg6*, is a coiled-coil protein in mammals that mediates autophagy.[11] A recent study has shown that *Beclin-1* is associated with class III phosphatidylinositol 3-kinase and generates phosphatidylinositol 3-phosphate, resulting in the initiation of autophagy.[12] It can also normalize chromosome congression and kinetochore assembly during mitosis.[13]

Vitrification is a fast-freezing technique that has become the most effective method to preserve human oocytes and embryos. Using highly concentrated cryoprotectants (CPAs), rapid freezing prevents the formation of ice crystals.[14] However, vitrification may disturb oocytes due to factors such as toxicity from high concentrations of CPAs, cold shock, and osmotic stress. Moreover, it may disturb organelles, hardens the zona pellucida, and affects gene expression. It was proposed that stabilization of cytoskeleton fiber during vitrification with stabilizers such as docetaxel could improve the cryotolerance of oocytes.[15] Docetaxel prevents depolymerization of microtubules by stabilizing their structure through binding with the β-subunit of tubulin of the microtubules, i.e., the vitrification-warming process improves the viability of oocytes.[16] The quality of vitrified-warmed oocytes was evaluated in terms of embryonic growth rate after fertilization, the condition of the meiotic spindle, DNA damage, generation of reactive oxygen species, and ultrastructural changes in oocytes.[17] Given the importance of autophagy in the viability and developmental competence of oocytes, the present study aimed to assess the impact of docetaxel on the survival, fertilization, and expression of *Atg5* and *Beclin-1* genes in vitrified oocytes.

Materials and Methods

The present experimental study was conducted in 2018 at the Stem Cells Technology Research Center, Shiraz University of Medical Sciences (Shiraz, Iran). The study and the animal care protocol for research were approved by the Ethics Committee of Shiraz University of Medical Sciences, Shiraz, Iran (IR.SUMS.REC.1395.S855).

Animals

Female (8-10 weeks old) and male (10-12 weeks old) Naval Medical Research Institute (NMRI) mice were used in the study. They were purchased from Royan Institute (Karaj, Iran) and housed under controlled temperature (22±2 °C), humidity of 40-50%, and 12:12 hours light:dark cycle. The mice had access to standard rodent chow (Behparvar®, Tehran, Iran) and water ad libitum.[18]

Oocyte Retrieval

To obtain the highest possible number of oocytes for vitrification, female mice were superovulated by intraperitoneal injection of 10 IU pregnant mare serum gonadotropin (PMSG; Gonaser, HIPRA, Spain) and 48 hours later by intraperitoneal injection of 10 IU human chorionic gonadotropin (HCG; Organon, Oss, The Netherlands). Aspiration of oocyte cumulus complexes from the antral follicle was done 12 to 15 hours after HCG administration. Incubation was performed for 30 to 60 seconds in a basal medium (G-MOPS™, Vitrolife, Sweden) supplemented with 300 µg/mL hyaluronidase (code: 4272, Sigma, UK). Identification of matured metaphase II (MII) oocytes was performed using a light microscope (Olympus,

Tokyo, Japan) and determined by the presence of the first polar body and the size of perivitelline space. Except for the control and vitrified oocytes, other oocytes were pre-incubated with docetaxel (Sigma Aldrich, Gillingham, Dorset, UK) at a concentration of 0.05 µM for 20 min.[19, 20]

Experimental Design

Mature oocytes were randomly assigned to five groups, namely (i) control group (n=133): untreated oocytes as fresh controls, (ii) docetaxel group (n=136): oocytes pre-incubated with docetaxel for 20 min, (iii) docetaxel+CPA group (n=146): oocytes pre-incubated with docetaxel for 20 min and exposed to CPAs without vitrification, (iv) docetaxel+vitrification group (n=138): oocytes pre-incubated with docetaxel for 20 min and vitrified, and (v) vitrification group (n=145): vitrified oocytes. Vitrification of oocytes was performed using a cryotop® container (KITAZATO Co., Tokyo, Japan).

Vitrification and Warming Solution

The vitrification solution was supplemented with 15% ethylene glycol together with 15% glycerol and 0.5 M sucrose (all from Sigma, USA) as basal medium. The equilibration solutions contained half of the CPAs of the vitrification solution without sucrose. Warming solutions (WS1, WS2, and WS3) were prepared by adding low concentrations of sucrose (1, 0.5, and 0.25 M, respectively) to the basal medium.

Vitrification and Warming

Vitrification: The denuded MII oocytes in batches of 15 were vitrified in a cryotop container according to protocols described in a previous study.[20] In brief, the oocytes in the basal medium were gradually exposed to the equilibration solution for 3 min and then incubated in the vitrification solution for 1 min. Each batch was loaded onto the cryotop container, and the excess solution was removed. The container with the oocytes was sealed, immersed in liquid nitrogen, and stored.

Warming: The cryotop containing oocytes was exposed to the warming solution in three steps, namely in WS1 for 1 min, WS2 for 3 min, and WS3 for 5 min. It was then transferred to a washing solution for 5 min and collected for further analysis (figure 1).

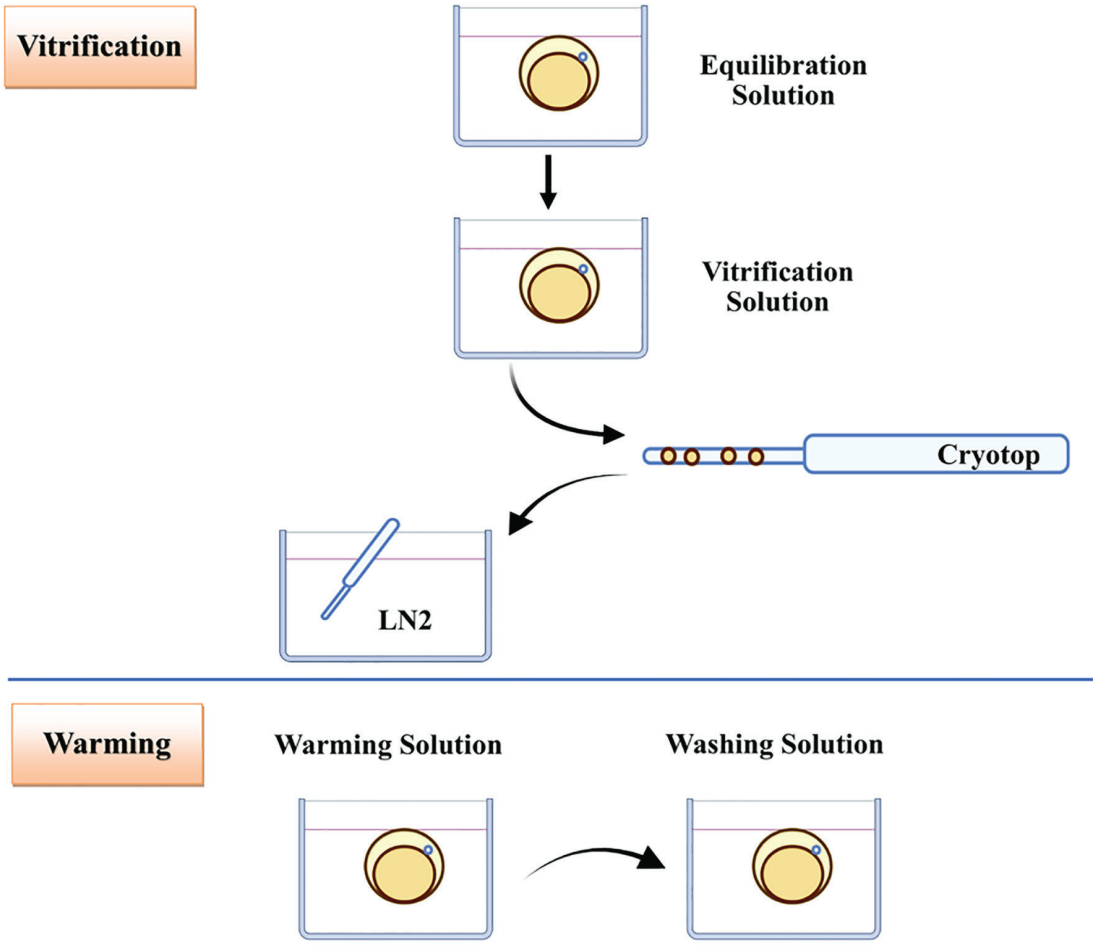

Figure 1: The vitrification-warming procedure is illustrated.

The complete vitrification process was performed at room temperature (25 ˚C) in accordance with the protocol described in previous studies.[14, 21]

In Vitro Fertilization

The fresh and vitrified-warmed oocytes were transferred to a pre-warmed G-IVF medium (Vitrolife, Sweden) covered with mineral oil (CAS: 8410; Sigma, USA). The cauda epididymis of adult male mice was excised, and several incisions were made to extract the sperms. The sperms were capacitated by incubation in a pre-warmed G-IVF medium drop in 5% CO_2 at 37 °C for 45 min. The final concentration of 2×10^6 spermatozoa/mL was added to each G-IVF medium containing 15 oocytes and incubated in 5% CO_2 at 37 °C for 5 hours. The pronuclei in each group were inspected after 24 hours under an inverted microscope (Nikon, Japan), and the formation of at least two pronuclei was considered as the fertilization rate.

RNA Extraction and Real-time Polymerase Chain Reaction (PCR)

Total cellular RNA was extracted from the vitrified and non-vitrified oocytes using an RNA extraction kit (CinnaGen Co., Tehran, Iran). The quantity and quality of RNAs were assessed using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) with 260/280 nm optical density ratio and 1% agarose gel. The cDNA was synthesized with 1000 ng of the total RNA using RevertAid™ first strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Quantitative real-time PCR (qPCR) was performed using Applied Biosystems StepOne™ and RealQ Plus 2x Master Mix Green (Ampliqon A/S, Odense, Denmark). Based on the mouse DNA sequences found in GenBank and BLAST query,[22] the sequence of primers was determined (table 1). β-actin was used as an internal control (housekeeping gene). Amplification signals of various samples were normalized with β-actin to determine the cycle threshold (Ct). The fold-change was determined using the $2^{-\Delta\Delta CT}$ method by comparing the activated versus controlled levels of mRNA.[23] All experiments were performed in triplicate.

Statistical Analysis

Data were analyzed using SPSS software (version 26.0) with one-way analysis of variance (ANOVA). Tukey's test was used to determine the difference between the mean value of the survival and fertilization of MII oocytes. Gene expression levels were analyzed using GraphPad Prism 9 software (GraphPad Software Inc. La Jolla, California, USA). The normality and homogeneity of the data were examined prior to data analysis. $P<0.05$ was considered statistically significant.

Results

Assessment of Oocytes Viability and Fertilization

The viability of the oocytes was assessed by comparing the morphology of each of the treatment groups with the control group (figure 2).

Table 1: Primer sequences used for the real-time polymerase chain reaction

Gene	Sequence	Size (bp)
M-*Atg5*	Forward: AACTGAAAGAGAAGCAGAACCA	105
	Reverse: TGTCTCATAACCTTCTGAAAGTGC	
M-*Beclin*-1	Forward: AATCTAAGGAGTTGCCGTTATAC	187
	Reverse: CCAGTGTCTTCAATCTTGCC	
M-β-*actin*	Forward: AGTGTGACGTTGACATCCGT	120
	Reverse: TGCTAGGAGCCAGAGCAGTA	

M: Mouse; bp: Base pair

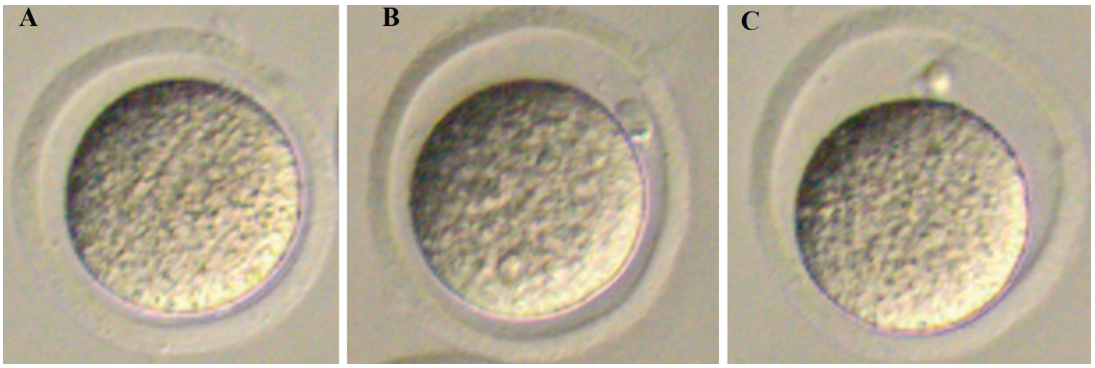

Figure 2: The morphology of vitrified metaphase II oocytes is shown after warming. (A) Control, (B) Vitrification, and (C) Docetaxel+vitrification.

Table 2: The survival and fertilization rate of the MII oocytes in the studied groups

Variables	Control	Docetaxel	Docetaxel+ cryoprotectant	Docetaxel+ vitrification	Vitrification	P value*
Survival of oocytes after vitrification	130/133 (97.79±0.73)	124/136 (91.15±1.23)	130/146 (88.99±1/27)	121/138 (87.68±0.43)	120/145 (82.75±1.33)	<0.001
Fertilization of oocytes (2-cell formation)	84/94 (89.47±2.003)	73/98 (83.86±1.55)	97/121 (80.22±0.83)	65/98 (66.26±1.45)	61/98 (62.26±2.15)	<0.001

Data are presented as the total number of three replicates in each group and expressed as mean±SEM (standard error of the mean). Two-cell formation was observed 24 hours after *in vitro* fertilization. *One-way analysis of variance (statistical significance: P<0.001).

Table 3: The results of pairwise comparison using Tukey's test

Variables	Survival of oocytes after vitrification		Fertilization of oocytes (two-cell formation)	
	Mean±SEM	P value	Mean±SEM	P value
Group I vs. Group II	6.64±1.5	0.004*	5.61±2.35	0.17
Group I vs. Group III	8.8±1.5	<0.001**	9.24±2.35	0.01*
Group I vs. Group IV	10.11±1.5	<0.001**	23.21±2.35	<0.001**
Group I vs. Group V	15.04±1.5	<0.001**	27.21±2.35	<0.001**
Group II vs. Group III	2.15±1.5	0.61	3.63±2.35	0.55
Group II vs. Group IV	3.46±1.5	0.19	17.59±2.35	<0.001**
Group II vs. Group V	8.4±1.5	<0.001**	21.59±2.35	<0.001**
Group III vs. Group IV	1.3±1.5	0.9	13.96±2.35	<0.001**
Group III vs. Group V	6.24±1.5	0.006*	17.96±2.35	<0.001**
Group IV vs. Group V	4.93±1.5	0.03*	3.99±2.35	0.46

*Significance at P=0.05; **Significance at P=0.001; Group I: Fresh control; Group II: Docetaxel; Group III: Docetaxel+CPA; Group IV: Docetaxel+vitrification; Group V: Vitrified oocytes

The viability of oocytes in all groups was compared to controls (tables 2 and 3). The survival rate of oocytes was significantly reduced in both the docetaxel+vitrification and vitrification groups compared to controls (P<0.001). However, there was no statistically significant reduction in the survival rate of oocytes in the docetaxel+CPA and docetaxel+vitrification groups compared to the docetaxel group (P=0.610 and P=0.190, respectively). The results showed a significant reduction in the fertilization rate in all treatment groups compared to controls. We found significant variations in the fertilization rate observed in the docetaxel group compared to the docetaxel+vitrification and vitrification groups (P<0.001). The two-cell formation rate after IVF was significantly lower in the docetaxel+CPA, docetaxel+vitrification, and vitrification groups than the controls (P<0.001).

Autophagy-related Gene Expression

The expression of *Beclin-1* and *Atg5* mRNA was examined in the vitrified-warmed and non-vitrified groups compared to controls. *Beclin-1* gene expression level was significantly higher in the docetaxel+vitrification (P=0.003) and

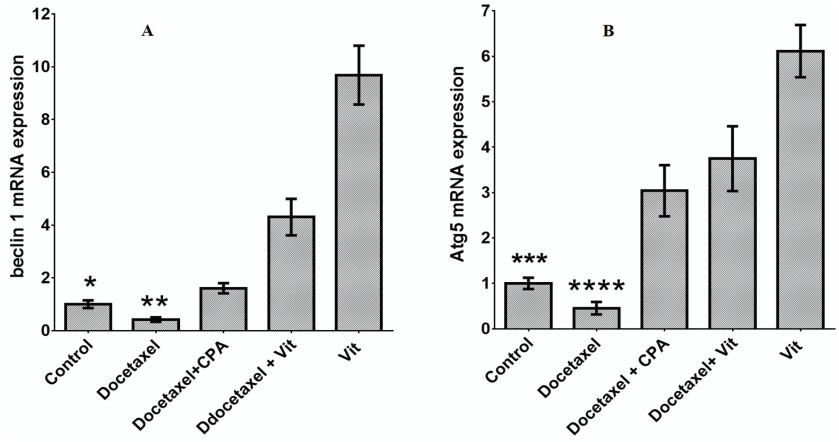

Figure 3: The expression levels of *Beclin-1* (A) and Atg5 (B) genes are shown in vitrified and non-vitrified MII oocyte groups. *Significant difference with the docetaxel+CPA (P=0.047), docetaxel+vitrification (P=0.003), and vitrification (P<0.001) groups. **Significant difference with the control (P=0.008), docetaxel+CPA (P<0.001), docetaxel+vitrification (P<0.001) and vitrification (P<0.001) groups. ***Significant difference with the docetaxel+CPA (P=0.007), docetaxel+vitrification (P=0.005), and vitrification (P<0.001) groups. ****Significant difference with the control (P=0.018), docetaxel+CPA (P=0.002) and docetaxel+vitrification (P=0.001) groups.

vitrification (P=0.001) groups than the controls. The results showed that pre-incubation of oocytes with docetaxel decreased *Beclin-1* gene expression levels in the docetaxel+CPA group compared to the docetaxel+vitrification group (P=0.004). As shown in figure 3, there was a significant decrease in *Beclin-1* gene expression level in the docetaxel group compared to controls (P=0.008). *Atg5* gene expression levels were also significantly higher in the vitrified groups (docetaxel+vitrification [P=0.005] and vitrification [P=0.001]) than the non-vitrified groups. The results showed that pre-incubation of oocytes with docetaxel decreased *Atg5* gene expression levels in the docetaxel+CPA group compared to the docetaxel+vitrification group (P=0.459). Besides, there was a significant decrease in *Atg5* gene expression levels in the docetaxel group compared to controls (P=0.001).

Discussion

The vitrified-warmed mouse oocytes exhibited autophagic activation, whereas docetaxel had a protective effect on the survival and fertilization of autophagy-related genes of the vitrified oocytes. Docetaxel also reduced *Atg5* and *Beclin-1* gene expression levels in vitrified compared to non-vitrified oocytes. The results showed that incubation of oocytes with docetaxel before vitrification inhibited the spindle abnormalities and chromosomal distribution during vitrification and led to the downregulation of autophagic activities. We found that without pre-treatment with docetaxel, the expression level of autophagy-related genes (*Atg5*, *Beclin-1*) was increased, leading to cell damage and subsequently induced autophagy. In line with our results, Bang and colleagues reported that the expression of *Atg5 and Beclin-1* increased in vitrified-warmed oocytes in response to the warm and cold stresses during vitrification.[24] Another study concluded that immature oocyte cryopreservation exhibited autophagic activation, with negative effects on viability, cleavage, and blastocyst formation rates.[2] Our findings showed that survival and fertilization rates in vitrified-warmed oocytes pre-incubated with docetaxel were higher than the untreated oocytes. Therefore, it is suggested that the viability of normal cytoskeleton fiber, cortical granule, and mitochondria after the vitrification-warming process might be due to cell metabolism, proliferation, and differentiation. In natural fertilization, there is a homogenous cortical distribution in MII oocytes.[25, 26] Docetaxel, as a microtubule inhibitor, stabilizes the cytoskeleton fiber by preventing the disassembly of tubulin during vitrification.[25] Docetaxel positively affects the rate and extent of tubulin binding, promoting microtubule stability. After vitrification and warming, recovery of the oocyte meiotic spindle was promoted and embryo development was enhanced. Thus, the vitrified-warmed oocyte had similar cleavage timing, cell number, and DNA methylation patterns as the fresh oocytes.[25, 27] Chasombat and colleagues showed that 0.05 µM docetaxel had no toxic effect on oocyte survival and fertilization. They also showed that incubation of bovine oocytes with 0.05 µM docetaxel before vitrification resulted in oocytes with normal metaphase plate configuration as well as higher survival rate, division rate, and blastocyst formation after fertilization.[19]

Autophagy is induced as the main response of cells to stress conditions such as starvation, temperature changes, or hypoxia. It is hypothesized that autophagy is induced in response to stress conditions during oocyte meiosis.[28] Lee and colleagues found that *Atg5* and *Atg6* were highly expressed at the 1-cell stage, and then decreased through the morula and blastocyst stages in a steady manner.[29] Cells exposed to freezing conditions are damaged due to cold shock and osmotic stress. A previous study reported that autophagy played a role in stallion sperm death during storage in a fridge.[30] Although CPAs may induce osmotic damage and result in toxicity, they also play a fundamental role in minimizing cell dehydration and preventing the formation of ice crystals.[31, 32] Other studies suggested that autophagy is induced in oocytes during the vitrification-warming process to recycle damaged organelles. However, inhibition of autophagy may lead to apoptosis, since there is a correlation between autophagy and apoptosis.[2, 33] Similarly, in our previous study,[21] we showed that the expression of B-cell lymphoma-extra large (Bcl-xl) significantly increased in vitrified mature oocytes compared to controls. This indicates the initiation of a defense mechanism by vitrified-warmed oocytes to prevent apoptosis. Among all *Atg* genes, *Atg5* plays a significant role in autophagy activation through *Atg12-Atg5* conjugate and LC3 lipidation. Some studies indicated that autophagy in mammalian cells is caused by the overexpression of *Atg* genes.[34, 35] In line with our results, Pyo and colleagues suggested that although overactivation of excessive autophagy might not always be beneficial, ectopic expression of *Atg5* in mice regulates autophagic flux.[36]

The main limitations of the study were budget and time constraints. In addition, we only focused on oocytes. The developmental competence of

blastocyst-stage embryos was not evaluated due to the lack of access to media with cultures of cells from the zygote stage to the blastocyst stage.

Conclusion

Mouse MII oocytes incubated with docetaxel prior to the vitrification-warming process had higher survival and fertilization rates than the untreated oocytes. The pre-incubation also reduced the activity of autophagy-related genes. These findings may have significance in assisted reproductive technology in terms of oocyte developmental competence after vitrification.

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Authors' Contribution

H.D: Study design and data analysis. N.HR: Data collection and interpretation. Z.Kh, M.D: Data analysis. All authors have contributed to the drafting and revising of the manuscript. They have read and approved the final manuscript and are responsible for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of Interest: None declared.

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