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Original Article

The effect of docetaxel on survival, fertilization rate and apoptosis-related genes mRNA expression in mouse metaphase II oocytes following vitrification

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Abstract

Background: Docetaxel is beneficial in oocyte cryopreservation. **Aims:** The effect of docetaxel, on the survival, fertilization rate and mRNA expression of apoptosis-related genes of vitrified mature oocytes was investigated. **Methods:** Mature oocytes were divided into eight experimental groups, including I) control, II) docetaxel, III) docetaxel + cryoprotectant agent 1 (CPA1), IV) docetaxel + CPA2, V) docetaxel + vitrification 1 (Vit1), VI) docetaxel + Vit2, VII) Vit1, and VIII) Vit2. The survival and fertilization rates, and the mRNA expression level of *Bcl-xl*, *Bax* and *caspase-3* as apoptosis-related genes were evaluated. **Results:** The survival rates in Vit1, and Vit2 groups were significantly lower than in the control group ($P<0.05$). The fertilization rates in docetaxel + Vit1, docetaxel + Vit2, Vit1, and Vit2 were significantly lower than the control, docetaxel, and related groups using docetaxel and CPAs. *Bax* expression was significantly increased in groups which oocytes vitrified. Also, its expression in the Vit2 group increased significantly in comparison to the docetaxel + Vit2 group. The expression of the *Bcl-xl* gene was downregulated in docetaxel + CPA2, docetaxel + Vit2 and Vit2 compared to docetaxel group. The *Bax/Bcl-xl* ratio significantly increased in docetaxel + CPA2, docetaxel + Vit1, docetaxel + Vit2, Vit1 and Vit2 groups compared to control, docetaxel and the docetaxel + CPA1 group. *Caspase-3* expression significantly increased in all six groups in comparison to the control, and docetaxel groups. Its expression significantly increased in the Vit1 and Vit2 groups in comparison with docetaxel + Vit1, and docetaxel + Vit2, respectively. **Conclusion:** Docetaxel ameliorates the damages to oocytes during vitrification by altering the expression of apoptosis-related genes and its effects are dependent on the vitrification solution used in cryopreservation of oocytes.

Key words: Apoptosis, Docetaxel, Fertilization, Oocytes, Vitrification

Introduction

Female fertility can be preserved by three methods, namely the cryopreservation of embryo, oocyte and ovary. Among them, oocyte cryopreservation is considered an important component of human assisted reproductive technology (Chian *et al*., 2014; Rahbar and Abbasi, 2020). Cryopreservation of human oocytes is an established method for fertility preservation in women of reproductive age and also can provide a chance in assisted reproduction treatment to increase the efficiency of cycles of *in vitro* fertilization (IVF) in patients who must make more than one attempt following an ovarian stimulation, to reduce patients' exposure to exogenous gonadotrophins (Esmail-Abad *et al*., 2014; Parsanezhad *et al*., 2017). The first outcomes for embryos cryopreservation extracted from studies on several mammalian species including domestic animals such as ovine (Moawad *et al*., 2011), porcine (Somfai *et al*., 2007) and bovine (Chasombat *et al*., 2015). Oocytes from the domestic species are full of cytoplasmic lipid droplets and also very delicate to chilling which lead to reduced viability following the slow cooling, therefore the vitrification method is more useful and applicable in these animals (Hwang and Hochi, 2014).

Vitrification was used for oocyte and embryo cryopreservation many years after sperm preservation. This method has advantages, such as being less time-consuming and more cost-effective (Sharma and Sharma, 2020). The rate of cooling in vitrification is increased by minimizing the cryoprotectant agents' (CPAs) solution volume or improving the heat conductivity of cryo-

devices (Dhali *et al*., 2009; Shams Mofarahe *et al*., 2017). Some chemical compounds are considered cryoprotectants, including ethylene glycol (EG), 1,2-propanediol (PROH) and dimethylsulphoxide (DMSO) as permeable, and sucrose as non-permeable, agents. The high concentrations of CPAs in vitrification can protect the cells against ice formation. However, the high concentrations of CPAs are toxic because they induce osmotic stress; thus, this negative effect can be reduced by using the CPA mixture (Ghorbani *et al*., 2012; Esmaeilzadeh *et al*., 2015; Shams Mofarahe *et al*., 2020). Vitrification is a physical process and an ultra-rapid technique, whereby highly concentrated CPA and a rapid cooling rate lead to glass-like solidification. Temperature fluctuation (cold and heat shock) and osmotic stress are critical factors in vitrification, which can lead to cell damage, including the destruction of microfilament, organelles, zona pellucida hardening, and chromosome damage (Almasi *et al*., 2009).

The mammalian oocytes are large in size and more sensitive to changes in their environment. In the process of vitrification, oocyte viability is affected by CPA-concentrations, exposure time to vitrification solution and temperature (Kuwayama, 2007; Morató *et al*., 2008; Roozbehi, 2013).

Vitrification technique might affect the apoptotic gene profile. Programmed cell death (apoptosis) and proliferation are regulated by the B-cell-lymphoma protein 2 (*Bcl-2*) gene family. The Bcl-2 family as cytoplasmic proteins are important apoptotic factors, subdivided into pro-apoptotic (*Bax*), and anti-apoptotic (*Bcl-2*) groups. The mitochondrial pathway of apoptosis is mediated by the *Bcl-2* family members which act as anti-apoptotic or pro-apoptotic mediators. Maintaining a balance between these factors is critical for cell protection against apoptosis (Xiao *et al*., 2006; Bakhtari *et al*., 2020). B-cell-lymphoma protein 2 is situated in the outer mitochondrial membrane and prevents apoptotic mitochondrial changes (Ebrahimi *et al*., 2010). *Bax*, as a death promoter, found in cytosol of the healthy mammalian cell can induce apoptosis. In a damaged cell, *Bax* is translocated from the cytosol to the mitochondria by oligomerization. The oligomeric forms of *Bax* might damage mitochondria by pore formation, which triggers the release of apoptotic factors. *Bax* causes cytochrome C to be released from the mitochondria as a pro-apoptotic factor that can activate the caspase pathway and cause cell death (Gharenaz *et al*., 2018; Ilani *et al*., 2018). *Caspase-3* as an executioner of caspase family members has a vital role in cell apoptosis, which causes downregulation of *Bax/Bcl-2*. *Caspase-3* is cleaved and activated by the beginning of the apoptosis (Hou *et al*., 2018). Docetaxel as a spindle fiber stabilizer plays a critical role in successful vitrification of oocytes. It stabilizes binding with the b-subunit of tubulin in the microtubules and prevents microtubule depolymerization during vitrification, consequently reducing cytoskeleton fiber (CSF) damage. Therefore, it improves oocyte viability after vitrification-warming (Xiao *et al*., 2006; Fu *et al*., 2009; Pitchayapipatkul *et al*., 2017). The damage of the CSF of oocytes during vitrification is the main cause of abnormal spindle configuration and reduced viability of frozen-thawed oocytes. As the results showed the stabilization of CSF before vitrification with docetaxel could be beneficial for reducing CSF damage in oocytes and increased viability. Chasombat *et al*. (2015) showed that oocytes viability were improved in the presence of the docetaxel after warming and subsequent cleavage and blastocyst formation after IVF. Dual roles of the docetaxel have been reported in various studies, for example Cristofani *et al*. (2018) were shown that the docetaxel can modulate or induce apoptosis. Also, we recently showed that mitochondrial activity decreases in the presence of docetaxel which less mitochondrial activity can lead to decrease apoptosis.

Therefore, since cryopreservation of oocyte is an essential process in assisted reproductive technologies and also for fertility preservation, this study was designed to assess the effects of docetaxel on survival, fertilization rate and apoptosis-related genes following vitrification of mouse metaphase II (MII) oocytes.

Materials and Methods

Animals

In this experimental study, 28 female (8-10 weeks old) and 16 male (10-12 weeks old) NMRI strain mice were purchased from Royan Institute (Karaj, Iran), and kept under 12 h light/dark cycle in controlled temperature (22±2°C) and humidity of 40-50% for two weeks to adapt to laboratory conditions. Standard rodent chow and water were available *ad libitum* (Council, 2011). All procedures were approved by the Animal Care and the local Ethics Committee of Shiraz University of Medical Sciences, Shiraz, Iran (IR.SUMS.REC.1395. S855).

Oocyte collection

The female mice were super-ovulated by intraperitoneal (i.p.) injection of 10 IU pregnant mare's serum gonadotropin (PMSG; Gonaser, HIPRA, Spain), followed 48 h later by another i.p. injection of 10 IU human chorionic gonadotropin (hCG; Organon, Oss, Netherlands). About 15 h post-HCG administration, the mice were sacrificed by cervical dislocation and the cumulus oocyte complexes (COCs) were isolated from the oviduct. The cumulus cells were removed by incubating COCs in hyaluronidase (Sigma, USA) for 1 min and also gentle pipetting.

Preparation of solutions

Vitrification solution (VS1) was supplemented with 15% EG (Sigma, USA) + 15% DMSO (Sigma, USA) + 0.5 M sucrose (Sigma, USA) in the base medium (G-MOPS™) (Vitrolife, Göteborg, Sweden). The concentration of CPAs in equilibration solution (ES_1) was half of each VS_1 with no sucrose. VS_2 was supplemented with 7.5% EG + 7.5% glycerol (Sigma,

USA) + 0.5 M sucrose in the base medium. The concentration of CPAs in ES_2 was half of each VS_2 with no sucrose. Warming solution [1, 2, 3] (WS_1, WS_2, and WS_3), were prepared by supplementation of base medium with reduced sucrose concentrations (1, 0.5 and 0.25 M, respectively).

Experimental design

The matured MII oocytes with the extruded polar body were carefully chosen and randomly divided into eight groups (n=18) including control: untreated oocytes; docetaxel: oocytes pre-incubated with docetaxel; docetaxel + cryoprotectant (CPA1): oocytes were pre-incubated with docetaxel for 20 min and then exposed to ES_1 and VS_1, but not inserted in liquid nitrogen (LN_2); docetaxel + CPA2: Oocytes were pre-incubated with docetaxel for 20 min and then exposed to ES_2 and VS_2, but not inserted in LN_2; docetaxel + vitrification (Vit1): oocytes were pre-incubated with docetaxel for 20 min and then exposed to ES_1 and VS_1, and inserted in LN_2; docetaxel + Vit2: oocytes were pre-incubated with docetaxel for 20 min and then exposed to ES_1 and VS_1 and inserted in LN_2; Vit1: oocytes exposed to ES_2 and VS_2 and inserted in LN_2, and Vit2: oocytes exposed to ES_2 and VS_2 and inserted in LN_2. Oocytes incubation with docetaxel (Sigma-Aldrich, USA) was at a concentration of 0.05 µM for 20 min (Chasombat *et al*., 2015).

Vitrification and warming procedure

After incubation of oocytes with docetaxel, oocytes were exposed to ES_1 for 3 min and VS_1 for 1 min in the docetaxel + CPA1 group and to ES_2 for 3 min and VS_2 for 1 min in the docetaxel + CPA2 without immersion in LN_2. The same protocol was done for oocytes of docetaxel + Vit1 and docetaxel + Vit2, and finally the oocytes were loaded on top of the Cryotop® (Kitazato Ltd., Tokyo, Japan) with the least amount of VS_1, immersed into LN_2 (Amidi *et al*., 2018), and stored for one week. In the Vit1 and Vit2 groups, the oocytes without treatment with docetaxel were exposed, respectively, to ES_1 for 3 min and VS_1 for 1 min, and to ES_2 for 3 min and VS_2 for 1 min, and finally placed on Cryotop® and cryopreserved in LN_2. For warming, the vitrified oocytes of docetaxel + Vit1, docetaxel + Vit2, Vit1, and Vit2 groups were placed in WS_1, WS_2, and WS_3, respectively, for 1, 3 and 5 min and then transferred into the handling medium for further analyses. The vitrification and warming process was completed at room temperature according to the protocol described in our previous studies (Khodabandeh Jahromi *et al*., 2010; Amidi *et al*., 2018).

Assessment of the survival rate of oocytes

The survival rate of all oocytes after experiments was determined morphologically under an inverted microscope (Olympus, Tokyo, Japan). The survival rate of oocytes in the control group was assessed after collecting them from oviducts, before any experiments. The MII oocyte with normal appearance, including intact zona pellucida and homogenous cytoplasm, without color change was considered a viable mature oocyte.

Assessment of fertilization rate

Sperm preparation

Spermatozoa were collected from mature male NMRI mice (10-12 weeks) by removing and chopping the cauda epididymides. Then sperms were capacitated by preincubation at 37°C and 5% CO_2 for 1 h in 200 µL of G-IVF medium drops (Vitrolife, Göteborg, Sweden) under mineral oil (Reproline, Rheinbach, Germany).

In vitro fertilization

The oocytes harvested from all groups were placed into 200 µL drops of a G-IVF medium under mineral oil and the final concentration of 2×10^6 spermatozoa/ml was added to the G-IVF medium. At 5 h post-insemination, oocytes were monitored by invert microscope and the percentage of 2 pronucleus (PN) formation was recorded to evaluate fertilization rate.

Real time polymerase chain reaction (RT-PCR)

The oocytes were analyzed by RT-PCR to evaluate the expression level of apoptosis genes (*Bax*, *Bcl-xl*, and *Caspase-3*) in all groups. RNA extraction kit (CinnaGen Co., Iran) was used to extract total RNA from oocytes after experiments. Then, the quantity and quality of RNA were assessed by the optical density ratio 260/280 nm (NanoDrop™, Thermo Fisher Scientific, Wilmington, DE, USA) and agarose gel electrophoresis (1%). Total RNA was adjusted to a concentration of 1000 ng/ml. The first-strand complementary DNA (cDNA) was synthetized by RevertAid™ first strand cDNA synthesis kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

mRNA expression was evaluated by quantitative RT-PCR (qRT-PCR) using Applied Biosystems Step-One, and Real Plus 2x Master Mix Green (Ampliqon A/S, Odense, Denmark). Apoptosis genes (*Bax*, *Bcl-xl*, and *caspase-3*) primers were designed based on the mouse DNA sequences, which were found in the Genebank Primer-BLAST Online Program (Ye *et al*., 2012) (Table 1). The β-actin housekeeping gene was also used as the internal control of the qPCR reactions. All samples were prepared for qPCR conditions and set at 94°C for 10 min followed by 40 cycles of 15 s at 94°C, 60 s at 58°C, and extension of 7 min at 72°C. The amplification signals of different samples were normalized to β-actin cycle threshold (Ct), and then the delta-delta CT ($2^{-\Delta\Delta CT}$) method was applied to compare mRNA levels of activated vs. controls, which was represented as a fold change in data analysis. All experiments of RT-PCR were done in triplicate.

Statistical analysis

Statistical analysis was carried out using SPSS software (v. 21, Chicago, IL, USA). One-way analysis of variance (ANOVA) and the post-hoc Tukey test were used to determine the differences among groups within a category (survival or fertilization). The mRNA expression was analyzed by GraphPad Prism software

(GraphPad Software, San Diego, CA, USA). The p-value was considered to be statistically significant at <0.05.

Table 1: The sequences of primers used for RT-PCR

Gene	Primer sequencing (5´-3´)	Size (bp)
M-B-actin-F	AGTGTGACGTTGACATCCGT	120
M-B-actin-R	TGCTAGGAGCCAGAGCAGTA	
M-Bax-F	AGCAAACTGGTGCTCAAGGC	230
M-Bax-R	CCACAAAGATGGTCACTGTC	
M-Caspase-3-F	TGACTGGAAAGCCGAAACTC	122
M-Caspase-3-R	AGCCTCCACCGGTATCTTCT	
M-Bcl-xl-F	GACAAGGAGATGCAGGTATTGG	124
M-Bcl-xl-R	TCCCGTAGAGATCCACAAAAGT	

RT-PCR: Real time polymerase chain reaction

Results

Oocytes viability

The survival rate of pretreated oocytes in all experimental groups is shown in Table 2. The survival rate of oocytes in Vit1 and Vit2 groups showed a significant decrease when compared to the control group (P=0.001 and P=0.004, respectively). The viability of oocytes in the docetaxel group and docetaxel + CPA1 and docetaxel + CPA2 was reduced in comparison with the control group but the difference was not statistically significant (P>0.05). The difference between the survival rate of oocytes among docetaxel + CPA1, docetaxel + Vit1 and Vit1 groups and among docetaxel + CPA2, docetaxel + Vit2 and Vit2 groups was not statistically significant (P>0.05).

Table 2: Survival and the fertilization rate of the MII oocytes in all groups

Variable	Survival rate of MII oocytes	Fertilization rate
Control	89.87 ± 2.32^{a}	78.76 ± 1.76^{a}
Docetaxel	84.13 ± 4.57^{a}	74.39 ± 3.16^{a}
Docetaxel + CPA1	84.36 ± 2.16^{a}	78.86 ± 4.35^{ac}
Docetaxel + CPA2	83.77 ± 3.97^{a}	76.15 ± 2.15^{ad}
Docetaxel + Vit1	85.42 ± 1.66^{a}	65.93 ± 2.72^{bd}
Docetaxel + Vit2	83.30 ± 4.19^{a}	64.51 ± 3.66^{bc}
Vit1	79.19 ± 6.87^{b}	65.84 ± 2.73^{bd}
Vit2	80.55 ± 2.47^{b}	71.20 ± 6.64^{bc}

MII: Mature oocytes, CPA1: Cryoprotectant agent 1, and ES_1: Equilibration solution 1. CPA1: Oocytes exposed to ES_1 and VS_1, but not inserted in LN_2, CPA2: Oocytes exposed to ES_2 and VS_2, but not inserted in LN_2, Vit1: Oocytes exposed to ES_1 and VS_1 and inserted in LN_2, and Vit2: Oocytes exposed to ES_2 and VS_2 and inserted in LN_2. Percentage of survived and fertilized MII oocytes after experiments expressed as mean±SEM. Values with different superscript letters ($^{a, b, a, d}$) are significantly different at P<0.05. Control: Untreated oocytes, and Docetaxel: Oocytes pre-incubated with docetaxel for 20 min

Rate of fertilization

As shown in Table 2, incubation of oocytes with docetaxel, and with docetaxel + CPA1 and docetaxel + CPA2 had no deleterious effect on fertilization rate compared to control group (P>0.05). The rate of fertilization in docetaxel + Vit1, docetaxel + Vit2, Vit1 and Vit2 was significantly lower than in the control group (P<0.001). Moreover, the fertilization rate decreased significantly in docetaxel + Vit1, docetaxel + Vit2, Vit1 and Vit2 in comparison to the docetaxel group (P<0.001). Fertilization rate was significantly higher in docetaxel + CPA1 than docetaxel + Vit1 and Vit1 groups (P<0.001) and in docetaxel + CPA2 than docetaxel + Vit2 and Vit2 groups (P<0.001). There was no significant difference between docetaxel + Vit1 and Vit1 groups (P=0.179) and between docetaxel + Vit2 and Vit2 groups (P=0.110).

Expression level of *Bax*, *Bcl-xl*, *Bax/Bcl-xl* ratio and *caspase-3*

As Fig. 1 shows, the expression of the *Bax* gene was significantly higher in docetaxel + Vit1, Vit1 and Vit2 in comparison to control (P<0.001, P<0.001, and P=0.0045, respectively) and the docetaxel group (P<0.001, P<0.001, and P<0.001, respectively). Also, *Bax* expression was significantly upregulated in docetaxel + Vit1 and Vit1 groups than in the docetaxel + CPA1 group (P<0.001). Level of *Bax* mRNA expression in the Vit2 group increased significantly in comparison to docetaxel + Vit2 group (P=0.048).

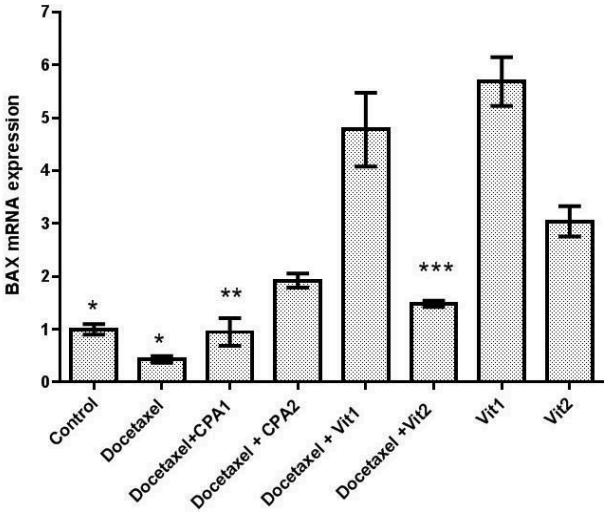

Fig. 1: The expression levels of *Bax* in different groups (P<0.05). CPA1: Cryoprotectant agent 1, CPA2: Cryoprotectant agent 2, Vit1: Vitrification 1, Vit2: Vitrification 2, ES_1: Equilibration solution 1 and VS_1: Vitrification solution 1. * Significant difference with docetaxel + Vit1, Vit1, and Vit2. ** Significant difference with docetaxel + Vit1, and Vit1. *** Significant difference with Vit2. CPA1: Oocytes exposed to ES_1 and VS_1, but not inserted in LN_2, CPA2: Oocytes exposed to ES_2 and VS_2, but not inserted in LN_2, Vit1: Oocytes exposed to ES_1 and VS_1 and inserted in LN_2, and Vit2: Oocytes exposed to ES_2 and VS_2 and inserted in LN_2. Control: Untreated oocytes, and Docetaxel: Oocytes pre-incubated with docetaxel for 20 min

The expression of *Bcl-xl* gene was significantly downregulated in docetaxel + CPA2, docetaxel + Vit2 and Vit2 compared to the docetaxel group (P=0.013, P=0.007, and P=0.021, respectively). There was no significant change among other groups (P>0.05) (Fig. 2).

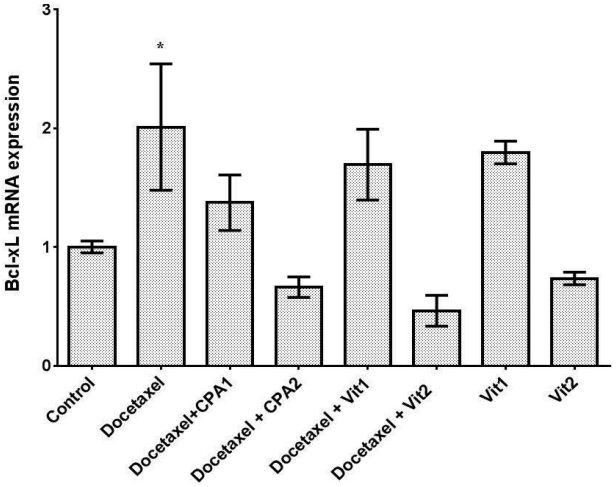

Fig. 2: The expression levels of *Bcl-xl* in different groups (P<0.05). CPA1: Cryoprotectant agent 1, CPA2: Cryoprotectnat agent 2, Vit1: Vitrification 1, Vit2: Vitrification 2, ES_1: Equilibration solution $_1$, and VS_1: Vitrification solution $_1$. * Significant difference with docetaxel + CPA2, docetaxel + Vit2, and Vit2. CPA1: Oocytes exposed to ES_1 and VS_1, but not inserted in LN_2, CPA2: Oocytes exposed to ES_2 and VS_2, but not inserted in LN_2, Vit1: Oocytes exposed to ES_1 and VS_1 and inserted in LN_2, and Vit2: Oocytes exposed to ES_2 and VS_2 and inserted in LN_2. Control: Untreated oocytes, and Docetaxel: Oocytes pre-incubated with docetaxel for 20 min

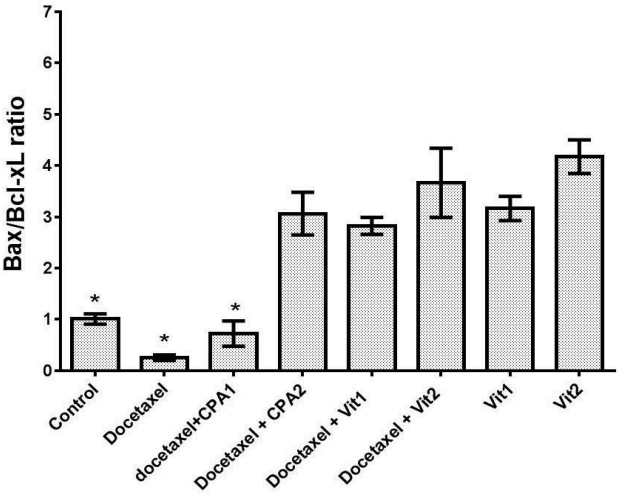

Fig. 3: The ratio of *Bax*/*Bcl-xl* in different groups (P<0.05). CPA1: Cryoprotectant agent 1, CPA2: Cryoprotectant agent 2, Vit1: Vitrification 1, and Vit2: Vitrification 2. * Significant difference with docetaxel + CPA2, docetaxel + Vit1, docetaxel + Vit2, Vit1, and Vit2. CPA1: Oocytes exposed to ES_1 and VS_1, but not inserted in LN_2, CPA2: Oocytes exposed to ES_2 and VS_2, but not inserted in LN_2, Vit1: Oocytes exposed to ES_1 and VS_1 and inserted in LN_2, and Vit2: Oocytes exposed to ES_2 and VS_2 and inserted in LN_2. Control: Untreated oocytes, and Docetaxel: Oocytes pre-incubated with docetaxel for 20 min

The *Bax/Bcl-xl* ratio increased in docetaxel + CPA2, docetaxel + Vit1, docetaxel + Vit2, Vit1 and Vit2 groups compared to control (P=0.001, P=0.009, P<0.001, P=0.001, and P<0.001, respectively) and the docetaxel group (P<0.001, P=0.001, P<0.001, P<0.001, and P<0.001, respectively). Also, the *Bax/Bcl-xl* ratio displayed significant increase in docetaxel + CPA2, docetaxel + Vit1, docetaxel + Vit2, Vit1 and Vit2 groups compared to docetaxel + CPA1 group (P<0.001, P=0.003, P<0.001, and P<0.001, respectively) (Fig. 3).

Regarding the expression of the *caspase-3* gene, in comparison to control and the docetaxel group, the expression level of this gene significantly increased in the other six groups (P<0.001) and its expression was significantly higher in the docetaxel + Vit1 and Vit1 groups than in the docetaxel + CPA1 group (P=0.001, and P<0.001, respectively). The *caspase-3* expression significantly increased in the Vit1 group when compared with the docetaxel + Vit1 group (P<0.001) and also in the Vit2 group in comparison to the docetaxel + Vit2 group (P=0.001) (Fig. 4).

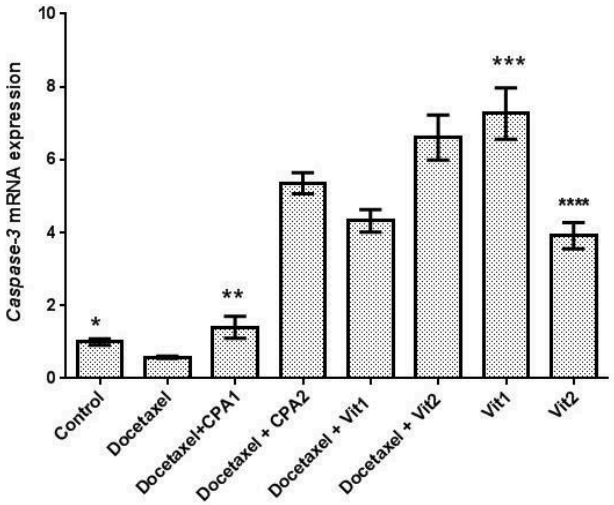

Fig. 4: The expression levels of *Caspase-3* in different groups (P<0.05). CPA1: Cryoprotectant agent 1, CPA2: Cryoprotectant agent 2, Vit1: Vitrification 1, and Vit2: Vitrification 2. * Significant difference with docetaxel + CPA1, docetaxel + CPA2, docetaxel + Vit1, docetaxel + Vit2, Vit1, and Vit2. ** Significant difference with docetaxel + Vit1, and Vit1. *** Significant difference with docetaxel + Vit1. **** Significant difference with docetaxel + Vit2. CPA1: Oocytes exposed to ES_1 and VS_1, but not inserted in LN_2, CPA2: Oocytes exposed to ES_2 and VS_2, but not inserted in LN_2, Vit1: Oocytes exposed to ES_1 and VS_1 and inserted in LN_2, and Vit2: Oocytes exposed to ES_2 and VS_2 and inserted in LN_2. Control: Untreated oocytes, and Docetaxel: Oocytes pre-incubated with docetaxel for 20 min

Discussion

In the current investigation, the effects of pretreatment of oocytes with docetaxel, as a microtubule stabilizer on survival and fertilization competence of oocytes following vitrification, were evaluated. Also, the changes in the mRNA levels of apoptotic genes, including *Bcl-xl*, *Bax*, *Bax*/*Bcl-xl* ratio, and *caspase-3* in the different groups of MII-oocytes, were determined.

Results demonstrated that oocyte vitrification using two different vitrification solutions, used in Vit1 and Vit2 groups, has detrimental effects on the survival rate of oocytes and even by pre-incubation of oocytes with docetaxel the fertilization rate is reduced. As results

show, the fertilization rate of oocytes preincubated in docetaxel and then exposed to CPA1 and CPA2 was higher compared to those oocytes which were vitrified demonstrating the detrimental effects of vitrification, even using docetaxel or without this substance, demonstrating that the harmful effects of vitrification are mostly also to preservation in low temperature. Regarding the influence of 0.05 μM of docetaxel, it is obvious that at the level of the mentioned dose, it has no deleterious effect on survival and fertilization rate of oocytes treated with it. In agreement with our study, Chasombat *et al*. (2015) showed that docetaxel at a concentration of 0.05 μM had no toxic effect on survival and fertilization of oocytes. They found that bovine oocytes pre-incubated with 0.05 μM docetaxel before vitrification were of higher quality and had a normal metaphase configuration as well as higher rates of survival, cleavage, and blastocyst formation after fertilization when compared with oocytes that were not pre-incubated before vitrification (Chasombat *et al*., 2015). In another study, it was found that a high concentration of docetaxel could induce high expression of pro-apoptotic proteins (*Bax* and *Bak*) and low expression of anti-apoptotic proteins (*Bcl-2* and *Bcl-xl*), leading to cell apoptosis (Domanska *et al*., 2012). Therefore, the dose of docetaxel was shown to have a direct effect on the viability and quality of oocytes.

Bcl-xl is an anti-apoptotic gene and *Bax* and pro-apoptotic gene. These genes have a prominent role in cell signaling and the regulation of apoptosis and can be evaluated in oocytes and also embryos produced *in vitro* (Yang and Rajamahendran, 2002). It is expected that the expression of *Bcl-2* as an anti-apoptotic gene occurs more in good quality oocytes and embryos in comparison to the *Bax* gene. *Bcl-xl* as an apoptotic inhibitor gene is a heterodimer with *Bax* and deactivates its effects (Luciano *et al*., 2004; Leon *et al*., 2013; De Bem *et al*., 2014).

The cell can be protected against apoptosis when the *Bcl-xl* expression increases. Moreover, the viability of vitrified/warmed oocytes could be evaluated by the expression of apoptotic genes. The *Bcl-2* family members controls the permeabilization of the mitochondrial outer membrane (Dhali *et al*., 2009). Equally, our study showed that *Bcl-xl* expression significantly increased in the vitrification group when compared to the control group, indicating that a defensive mechanism is activated in vitrified/warmed oocytes to prevent apoptosis damage.

It was shown that numerous stress factors activate or inhibit *Bcl-2* family proteins, including heat shock, γ and ultraviolet (UV) irradiation, nutrient deprivation, viral infection, hypoxia and amplified intracellular calcium concentration (Bogdał *et al*., 2013). It was also illustrated that internal stress and extracellular signals can increase *Bcl-xl* protein levels (Xu *et al*., 2011; Shamas-Din *et al*., 2013).

Another study indicated that changes in expression of *Bax*, *Bcl-2*, and *p53* genes in the vitrified embryos correlate with developmental competency (Ebrahimi *et al*., 2010).

The results in the present study showed that the fertilization rate of docetaxel pre-treated oocyte was higher than non-treated groups; moreover, the expression level of *Bax* as a pro-apoptotic gene increased in vitrified oocytes compared to those non-vitrified. The difference in *Bax* expression was also noted between docetaxel + Vit2 and Vit2 groups confirming the critical role of docetaxel in the conservation of oocyte to apoptosis initiation due to the inhibition effect of docetaxel in the mRNA expression of this apoptotic gene. It showed that oocytes in Vit1 were more susceptible to apoptosis than Vit2 and docetaxel could not rescue oocytes in Vit1 from harmful effects of vitrification.

Caspases are divided into initiator caspases such as *caspase-8* and *-9* and executioner caspases such as *caspase-3* or *-7*. They are members of a family of cysteine proteases. The cell sensitivity to apoptosis-inducing agents increased in the presence of *caspase-3* (Sharifi *et al*., 2015). Furthermore, in the current study, although the expression of *caspase-3* increased in all groups compared to the docetaxel and control groups, in oocytes treated with docetaxel before vitrification in both Vit1 and Vit2 groups, *caspase-3* expression significantly decreased the display of beneficial effects of docetaxel in reducing apoptosis promotion in oocytes (Chian *et al*., 2014).

Shahedi *et al*. (2017) reported that the *Bax/Bcl-2* ratio might be an important factor for the determination of cell survival or apoptosis. They reported that vitrification induced oxidative stress and led to an increase in the ratio of pro-apoptotic to anti-apoptotic mRNAs (Shahedi *et al*., 2017). These findings are similar to our study that showed an increased expression of the *Bax*/*Bcl-xl* ratio in all vitrification groups, while it decreased in the docetaxel group.

Sharifi *et al*. (2015) assessed the effects of doxorubicin on *Bcl-xl* and the *Bax* expression ratio in inducing apoptosis *in vitro*. They showed that *Bax/Bcl-xl* ratios can regulate the fate of a cell rather than the exact concentration of either *Bax* or *Bcl-xl* (Sharifi *et al*., 2015). Additionally, other researchers indicated that overexpressing the *Bax* gene displayed the tendency of oocyte towards apoptosis. It was found that though the *Bax/Bcl-xl* ratio was high in vitrified/warmed oocytes, other factors including external stimuli, internal defects and activation of other apoptotic-related genes would affect the fate (survival or death) of oocytes (Anchamparuthy *et al*., 2010; Abdollahi *et al*., 2013). Also, it has been demonstrated that pretreatment of oocytes with docetaxel before vitrification could increase the activity of mitochondrial genes correlated to the high quality and developmental competence of vitrified/warmed oocytes (Dehghani *et al*., 2019). Taken together, the results suggested that pretreatment of oocytes with docetaxel before vitrification has beneficial effects on the reduction of the detrimental effects of vitrification on oocytes. Also, it should be mentioned that its effects were more obvious in the Vit1 group; therefore, docetaxel effects are related to the vitrification protocol used. Our findings confirmed that pretreatment

of oocytes with docetaxel before vitrification can reduce damage by affecting on expression of pro-apoptotic and anti-apoptotic genes of vitrified/warmed oocytes and inhibition of cell fate towards apoptosis. Therefore, before vitrification, pre-treatment of oocytes with docetaxel can significantly decrease damages to oocytes. These data can be added to the literature targeting human assisted reproductive technology.

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Conflict of interest

The authors declare that there is no financial or non-financial conflict of interest in the publication of this manuscript.

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