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Comparison of apoptosis pathway following the use of two protocols for vitrification of immature mouse testicular tissue

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ABSTRACT

Our objective was to evaluate the apoptosis incidence in immature mouse testicular tissue after two different protocols of vitrification and short-term culture. Testes of 7-day-old Naval Medical Research Institute mice were isolated and distributed into control and vitrification groups. In vitrification 1 group, testes were vitrified using a combination of ethylene glycol and DMSO in three steps, and in vitrification 2 group, testes were vitrified using a combination of ethylene glycol and sucrose in five steps. Then, fresh and vitrified-warmed testis fragments were cultured for 20 hours. Morphology, cell viability, apoptosis incidence, and apoptosis gene expression (*BAX*, *BCL2*, *Caspase 3*, *Fas*, *Fas ligand*, *p53*) were evaluated at 0, 3, and 20 hours of culture by light microscopy, flow cytometry, and real-time polymerase chain reaction, respectively. Significant decrease of early apoptosis (annexin V+/PI− cells in vitrification 1 and 2 groups at 0 hours of culture, 37.34 ± 0.91 and 30.72 ± 2.2, and at 20 hours of culture, 1.46 ± 0.28 and 0.76 ± 0.11, respectively), increase of late apoptosis (annexin V+/PI+ cells in vitrification 1 group at 0 hours of culture, 14.46 ± 0.86, and at 20 hours of culture, 37.18 ± 2.34), and *BAX*/*BCL-2* ratio (in vitrification 1 and 2 groups at 0 hours of culture, 7.31 ± 0.31 and 6.83 ± 1.38, and at 20 hours of culture, 24.08 ± 4.32 and 9.35 ± 1.91, respectively) were observed in vitrification groups during culture period. *Caspase 3* expression was significantly decreased in all groups after 3 hours of culture (in control, vitrification 1, and vitrification 2 groups at 0 hours of culture, 1.00 ± 0.0, 1.56 ± 0.09, and 0.79 ± 0.06, and at 20 hours of culture, 0.37 ± 0.0, 0.96 ± 0.10, and 0.12 ± 0.03, respectively). Expression of *p53* was significantly lower in vitrification 1 (0.32 ± 0.02) and control (0.50 ± 0.03) groups in 20 hours of culture as compared with vitrification 2 (0.88 ± 0.14) group. *Fas* (in vitrification 1 and 2 groups at 0 hours of culture, 2.29 ± 0.23 and 1.14 ± 0.15, and at 20 hours of culture, 12.43 ± 0.46 and 6.7 ± 0.48, respectively) and *Fas Ligand* (in vitrification 1 and 2 groups at 0 hours of culture, 1.2 ± 0.28 and 5.24 ± 0.32, and at 20 hours of culture, 21.75 ± 2.00 and 25.82 ± 2.15, respectively) expressions significantly increased in vitrification groups after 20 hours of culture. Although both vitrification protocols cause cell death via apoptotic and necrotic pathway, it seems that vitrification 1 protocol induces cell death more via apoptotic pathway than via necrosis. The apoptosis incidence after vitrification may have occurred independent of *p53*.

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1. Introduction

Because of the advancements in cancer research, the life expectancies of young boys with cancer have been increasing [1]. Unfortunately, such patients often have to struggle with impaired fertility, caused by the disease itself or by the treatment processes [2]. Cryopreservation of testicular tissue is one of the noteworthy methods that has been used for fertility preservation [3]. Curaba et al. [4] showed that mouse testis tissue could be well preserved by vitrification and slow freezing. Another recent research showed that vitrification of testicular tissue is a time- and cost-efficient strategy to preserve spermatogonial stem cells [5].

Although vitrification of testicular tissue is considered as an appropriate and successful technique for fertility preservation in different species [6], the occurrence of inappropriate molecular and structural alterations during preservation is undeniable [7,8]. Apoptosis that happens after cryopreservation and culture is one of the important molecular alterations which caused the integrity reduction of testis [8].

Extrinsic and intrinsic pathways are involved in testicular germ cell death [9]. In the extrinsic pathway, activation of death receptors (such as Fas, TNF, etc.) causes activation of initiator Caspase 8 [10]. On the other hand, the intrinsic or mitochondrial pathway is activated by BCL2 family members that cause changes in mitochondrial membrane permeability and release of cytochrome c into the cytosol. Cytochrome c is involved in the formation of apoptosome complex with apoptotic protease activating factor 1 and causes activation of initiator Caspase 9 [11]. Both initiator caspases that are involved in intrinsic and extrinsic pathways activate executioner Caspase 3 and advance the apoptosis incidence further [12].

P53, tumor suppressor protein, is a sensor of genotoxic stress that is responsible for regulating the transcription of genes associated with cell cycle arrest, DNA repair, and apoptosis [13]. Research in 2008 showed increase of apoptotic cells in frozen testicular cells after 20 hours of culture [14].

In the present study, we have evaluated the effects of two different protocols of testis vitrification on tissue integrity, cell death, and expression of apoptotic genes during 20 hours of culture.

2. Materials and methods

2.1. Animals and study design

Forty-five immature male Naval Medical Research Institute mice (7-day-olds) were handled according to the Ethical Guideline of Royan Institute (Tehran, Iran). Their testicular tissues were isolated and randomly distributed into three experimental groups: Fresh control tissue (Cont), vitrification 1 (Vit 1), and vitrification 2 (Vit 2) groups (Fig. 1).

Experiments were replicated six times for cell viability and apoptosis assessment and three times for gene expression and morphology evaluation.

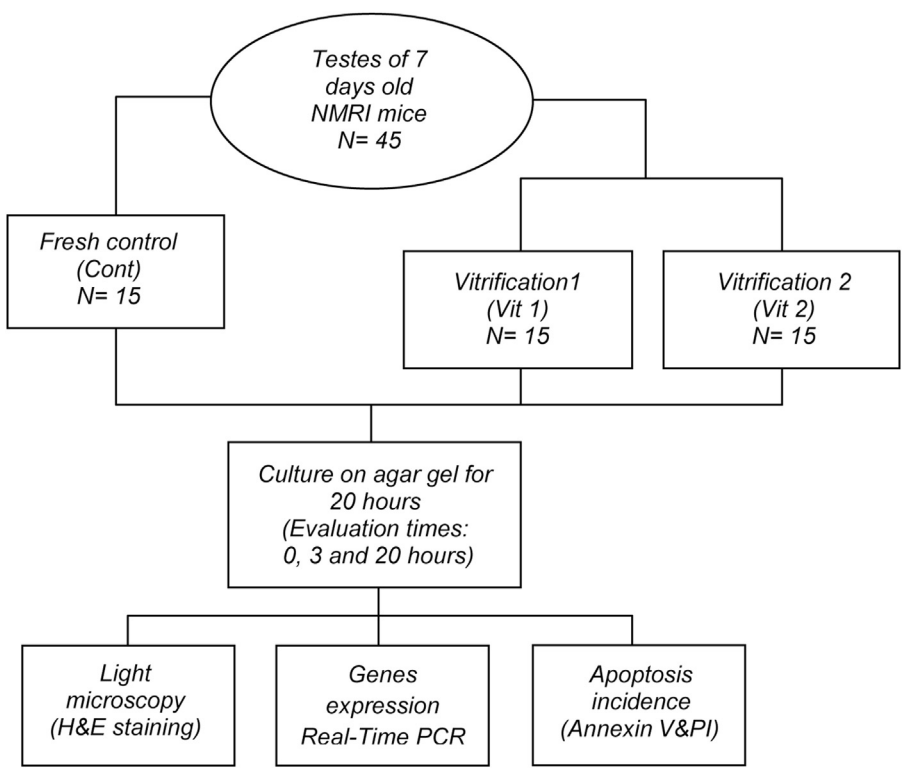

Fig. 1. Outline of the experimental procedure.

2.2. Vitrification

Basic medium (BM) in each vitrification solution was composed of Dulbecco's modified eagle medium (Gibco, Invitrogen, Carlsbad, CA, USA) and 20% FBS (Gibco, Grand Island, NY, USA). Vitrification solution 1 (Vit 1) was composed of BM, 2.8 mol/L DMSO (Sigma–Aldrich, St Louis, MO, USA), and 2.8 mol/L ethylene glycol (EG, Sigma–Aldrich). Each testis was sectioned longitudinally into two pieces (each piece: approximately 3.5 mm^3). Pieces in Vit 1 group were dehydrated by increasing concentrations of Vit 1 solution: 25% (5 minutes), 50% (10 minutes), and 100% (10 minutes) on ice [8], and in Vit 2 group, vitrification was performed according to the study by Gouk et al. [5] with slight modification in exposure times. In this group, testicular pieces were first dehydrated in 10%, 17.5%, and 25% of EG in BM for 3, 2, and 5 minutes, respectively. Further dehydration was done by 32.5% EG + 0.3 mol/L sucrose for 9 minutes and 40% EG + 0.6 sucrose for 9 minutes. Then, on the basis of the minimum essential medium rule, testicular tissue pieces were put on sterile surgical gauze to remove excess vitrification medium. They were then transferred to cooled cryovials and immersed in liquid nitrogen and preserved until warming time.

2.3. Warming

Warming for both vitrification groups was done according to the study by Curaba et al. [8]. Vitrified tissues were washed in warming solution 1 (W1; BM + 1 mol/L sucrose) for 1 minute, followed by washing for 5 minutes in W2, W3, and W4 solutions (0.5, 0.25, and 0.125 mol/L sucrose in BM), respectively [8].

2.4. In vitro culture

Liquid–gas interphase culture method was applied for *in vitro* tissue culture. Testicular pieces were divided again, and each fragment (approximately 1.75 mm^3) was cultured for 20 hours at 34 °C in a humidified atmosphere with 5% CO_2 in air on 0.15% of agar gel (Sigma–Aldrich) that was prepared according to Sato et al. [15]. RPMI (Gibco, Grand Island, NY, USA) with 10% Knockout Serum Replacement (Gibco, Invitrogen, Paisley, UK) was used as a culture medium.

2.5. Morphological evaluation

Testes were fixed in Bouin fixative for 24 hours and formaldehyde for 48 hours [7]. Tissues were dehydrated in ascending concentrations of ethanol, embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin. Testicular morphology was assessed by light microscopy. The morphological changes of fresh control and vitrified-warmed testis sections during culture period were assessed semiquantitatively based on the study by Milazzo et al. [14] in nucleus and epithelium level as follow:

(1) They were scored as 0, 1, and 2 if recognition of spermatogonia nuclei and sertoli cells was respectively easy, difficult, and impossible. (2) In nucleoli observation, 0 was related to the tubules with visible nucleoli in >40% of cells and one was related to indiscernible nucleoli (in the case of pyknotic nuclei present in large numbers and very condensed). (3) Absence of condensed nuclei or presence of only one condensed nuclei in tubule was scored as 0; if <40% of nuclei were condensed, it was scored as 1, and as 2 if >40% were pyknotic. Therefore, if total nuclei score was 0, it meant the absence of nuclei alteration and if it was 5, it meant the worst nuclei morphology.

The epithelium was scored too. (1) Absence of cells detachment from the basement membrane, partial detachment of cells, and total cell detachment or observed on >75% of the circumference were scored as 0, 2 and 3, respectively. (2) Absence, slight moderation, and severe change of shrinkage and gap formation in tubules were scored as 0, 2, and 3, respectively. Therefore, epithelium morphology was also scored from 0 to 5. Finally, the global score for each seminiferous tubule section was the sum of nuclei and epithelium score and was between 0 and 10. For each quarter of testis, the mean percentage of 30 seminiferous tubule global score was calculated.

Accordingly, the seminiferous tubule structure was judged as being good, moderately damaged, and damaged if scores were between 0 and 3, 3 to 6, and 6 to 10, respectively.

2.6. Cell isolation

Cell isolation was performed in all experimental groups at the 0th (just after warming), 3rd, and 20th hours of culture. Testis fragments were first placed in PBS containing 1 mg/mL Collagenase IV (Gibco, Grand Island, NY, USA) and 1 μg/ml DNase I (Sigma–Aldrich) and dissected mechanically. Then, the suspension was incubated for 20 minutes at 37 °C in a humidified atmosphere with 5% CO_2 in air. Finally, PBS^- was added for stopping enzymatic reaction, and the mixtures were filtered and washed.

2.7. Flow cytometric assessment

Apoptosis incidence was evaluated using an apoptosis kit (IQ Product, Groning, The Netherlands) containing fluorescein isothiocyanate–labeled annexin V–binding buffer and propidium iodide (PI). Annexin V was used for detection of externalized phosphatidylserine and PI was used for the identification of dead cells. Cell populations were counted as follows: annexin V−/PI− as live cells, annexin V−/PI+ as necrosis, annexin V+/PI− as early apoptosis, and annexin V+/PI+ as late apoptosis. Staining of cell suspensions was performed in accordance with recommended procedure by the manufacture. Briefly, 10 μL annexin V fluorescein isothiocyanate was added to 100 μL of cell suspension containing 10^6 cells/mL in calcium buffer and incubated on ice for 20 minutes, then washed by calcium buffer, stained by PI, and analyzed by flow cytometry method.

2.8. Gene expression

2.8.1. RNA extraction

Testicular fragments in control and vitrification groups were collected at 0, 3, and 20 hours of culture and stored in

RNA later reagent (Ambion, USA). They were stored at −70 °C until RNA extraction time. Total RNA was extracted from testes using a manual method described by Trizol (Sigma–Aldrich) as follows: small testicular fragments were added to Trizol, homogenized, incubated for 5 minutes on ice, centrifuged (12,000 rpm, 15 minutes, 4 °C) after adding the chloroform (Merck, Germany), and then cooled isopropanol (Merck, Germany) was added. The mixture was incubated for 60 minutes at −20 °C and centrifuged (12,000 rpm, 15 minutes, 4 °C) again. Finally, RNA pellets were washed with 70% ethanol, air dried, and dissolved in diethylprocarbonation-treated water. The optical density was determined at 260 nm.

2.8.2. Primer design

Specific primers for *BAX*, *BCL2*, *Fas*, *Fas ligand*, *Caspase 3*, and *p53* were designed by primer design software (Alel ID-Primer Biosoft, Palo Alto, CA, USA).

2.8.3. cDNA synthesis

cDNA was synthesized using RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, Leon-Rot, Germany) and random hexamers according to the manufacturer's instructions.

2.8.4. Real-time polymerase chain reaction

Each polymerase chain reaction (PCR) reaction mixture consisted of 2 μL of cDNA, 6 μL dH_2O, 10 μL Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK), and 1 μL of each sense and antisense primers (5 pmol). The PCR reaction mixture was prepared in duplicate, and the system (Applied Biosystems StepOne; UK) was run according to the following protocol: stage 1: 95 °C for 10 minutes; stage 2: 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute; and stage 3: 95 °C for 15 seconds, 60 °C for 15 seconds, and 95 °C for 15 seconds. The quantity of gene expression was normalized by GAPDH (housekeeping gene) and formula of $2^{-\Delta\ (\Delta CT)}$ was used to calculate the quantification of normalized target gene in each experiment. Comparison of relative genes expression between control and vitrification groups was made with ΔΔCT method. The 0 hours of culture system in the control group was considered as the zero level for calculation of ΔΔCT. $2^{-\Delta\Delta CT}$ Values were calculated for each gene on each time of evaluation in experimental groups [16]. To ensure accurate products and the absence of DNA contamination, "No template control" sample was run simultaneously with test samples. To test the appearance of the PCR products, the samples were additionally run in 2% agarose gel and confirmed with obtained melt curves in the setting-up stage.

2.9. Statistical analysis

Data were presented as mean ± SEM, and the statistical package for social science version 16.0 (SPSS, Chicago, IL, USA) was used to run two-way ANOVA on necrosis, early apoptosis, late apoptosis, living cells, and genes expression data in two distinct directions: (A) at each time of culture period between groups and (B) in each group during the culture period. For consideration of significance levels, Tukey post hoc test was applied, and the significant level was considered at $P < 0.05$.

3. Results

3.1. Morphological evaluation

Semiquantitative evaluation of testicular tissues in control and vitrification groups was performed at 0, 3, and 20 hours of culture. Structural integrity of testicular sections in Vit 1 group at 0 hours of culture was comparable with fresh control tissues and mild alterations such as the presence of the low numbers of pyknotic nuclei in some seminiferous tubules was observed. At 0 hours of culture, 95.33 ± 1.45 and 87.53 ± 1.25 of seminiferous tubules in fresh control and Vit 1 groups were respectively judged as being good (Fig. 2A, B). However, at this time, only 21.66 ± 2.51 of seminiferous tubules were assessed as good in Vit 2 group, and alterations of testicular integrity, presence of numerous pyknotic nuclei, rupture of some seminiferous tubules and interstitial tissues, and detachments of cells from basal lamina were observed in this group (Fig. 2A, B). Deterioration of tissue integrity in central areas as disruption of interstitial tissues and seminiferous tubules commenced in all groups at 3 hours of culture, and it intensified during the final hours of culture. At 3 hours of culture, 87.53 ± 1.25, 65.97 ± 1.39, and 21.36 ± 3.45 of seminiferous tubules in control, Vit 1, and Vit 2 groups were respectively considered as good (Fig. 3A, B). At 20 hours of culture, 63.33 ± 2.03, 68.87 ± 2.94, and 24.13 ± 3.66 of seminiferous tubules in Vit 1, Vit 2, and control groups were respectively considered as damaged (Fig. 4A, B).

3.2. Flow cytometric assessment

3.2.1. Living cells

A: At 0, 3, and 20 hours of culture, mean percentage of living cells decreased significantly in both vitrification groups compared to the control group, and at 0 and 3 hours of culture, it was significantly higher in Vit 1 than in Vit 2 group.

B: Mean percentage of living cells decreased significantly in all experimental groups during culture period (Table 1).

3.2.2. Early apoptotic cells

A: Although early apoptosis increased in Vit 1 and Vit 2 groups as compared to the control group at 0 hours of culture, at 20 hours of culture time, greater increase of early apoptosis was observed in the control group than the other groups.

B: During the culture period, the mean percentage of early apoptotic cells decreased significantly in both vitrification groups, whereas a significant increase of early apoptosis was indicated in control group at 3 and 20 hours of culture (Table 1).

3.2.3. Late apoptotic cells

A: At 0 and 3 hours of culture, mean percentage of late apoptotic cells was significantly higher in Vit 1 and Vit 2 groups than the control. The highest increase was seen in

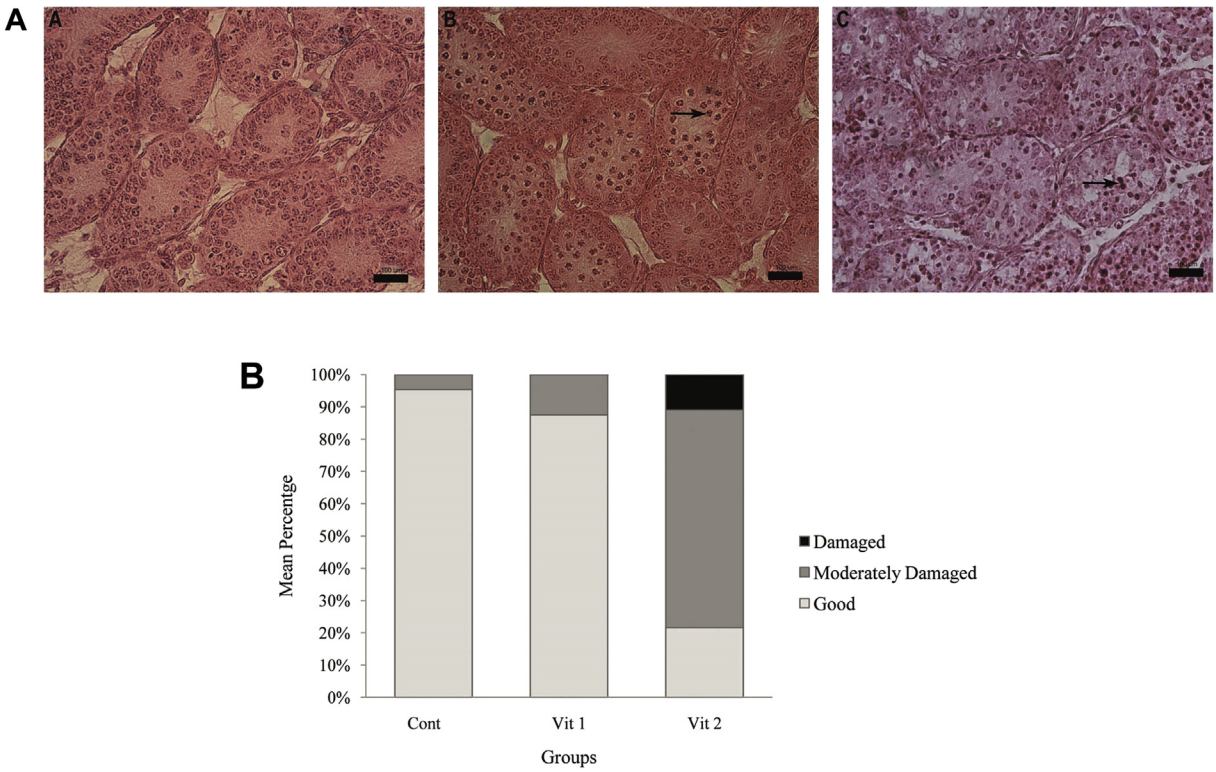

Fig. 2. (A) Hematoxylin-eosin staining of cultured testis fragments at 0 hours of culture in all experimental groups. (A, B, C) Histologic sections of fresh control (Cont) and vitrified-warmed testis fragments in vitrification 1 (Vit 1) and vitrification 2 (Vit 2) groups, respectively. Black arrow: pyknotic nuclei, original magnification ×400. (B) Semiquantitative data (good, moderately damaged, and damaged seminiferous tubules) of cultured testis fragment at 0 hours of culture in all experimental groups.

Vit 1 group. This variable was significantly higher in Vit 1 and control groups than in Vit 2 group at 20 hours of culture period.

B: During the culture period, late apoptosis in control and Vit 1 group was increased continuously from 0 to 20 hours of culture, whereas in Vit 2 group, it increased till 3 hours of culture and then decreased till 20 hours of culture, and finally, it reached the same point as 0 hours of culture (Table 1).

3.2.4. Necrotic cells

A: At 3 and 20 hours of culture, mean percentage of necrosis was significantly higher in both vitrification groups than the control group, and at 0 and 20 hours of culture, the highest increase of necrotic cells was observed in Vit 2 group, whereas at 3 hours of culture, it was the highest in Vit 1 group.

B: Significant increase of necrosis was seen in both vitrification groups at 20 hours of culture compared to 0 hours of culture (Table 1).

3.3. Gene expression

3.3.1. BAX

A: At 0 and 20 hours of culture, Vit 1 group showed the highest increase of *BAX* expression among all groups. At 3 hours of culture, significant increase of *BAX* was found in Vit 1 and Vit 2 groups compared to the control group.

B: Although relative expression of *BAX* increased significantly from 0 to 20 hours of culture in Vit 1 group, it decreased significantly in the Vit 2 group (Fig. 5).

3.3.2. BCL2

A: At 0, 3, and 20 hours of culture, relative expression of *BCL2* was significantly lower in both vitrification groups than in the control group (Fig. 5).

B: Within culture hours, in both vitrification groups, relative expression of *BCL2* decreased in 20 hours of culture compared to 0 hour of culture (Fig. 5).

3.3.3. Ratio of BAX to BCL2

A: At all times of culture, ratio of *BAX* to *BCL2* was higher in vitrification groups than in the control.

B: Nonsignificant increases in all experimental groups were observed at 20 hours of culture compared to 0 hours of culture (Fig. 6).

3.3.4. Caspase 3

A: Relative expression of *Caspase 3* was significantly higher in Vit 1 group than the other groups at all times of culture.

B: Significant decrease of *Caspase 3* expression was observed in all experimental groups up to 3 hours of

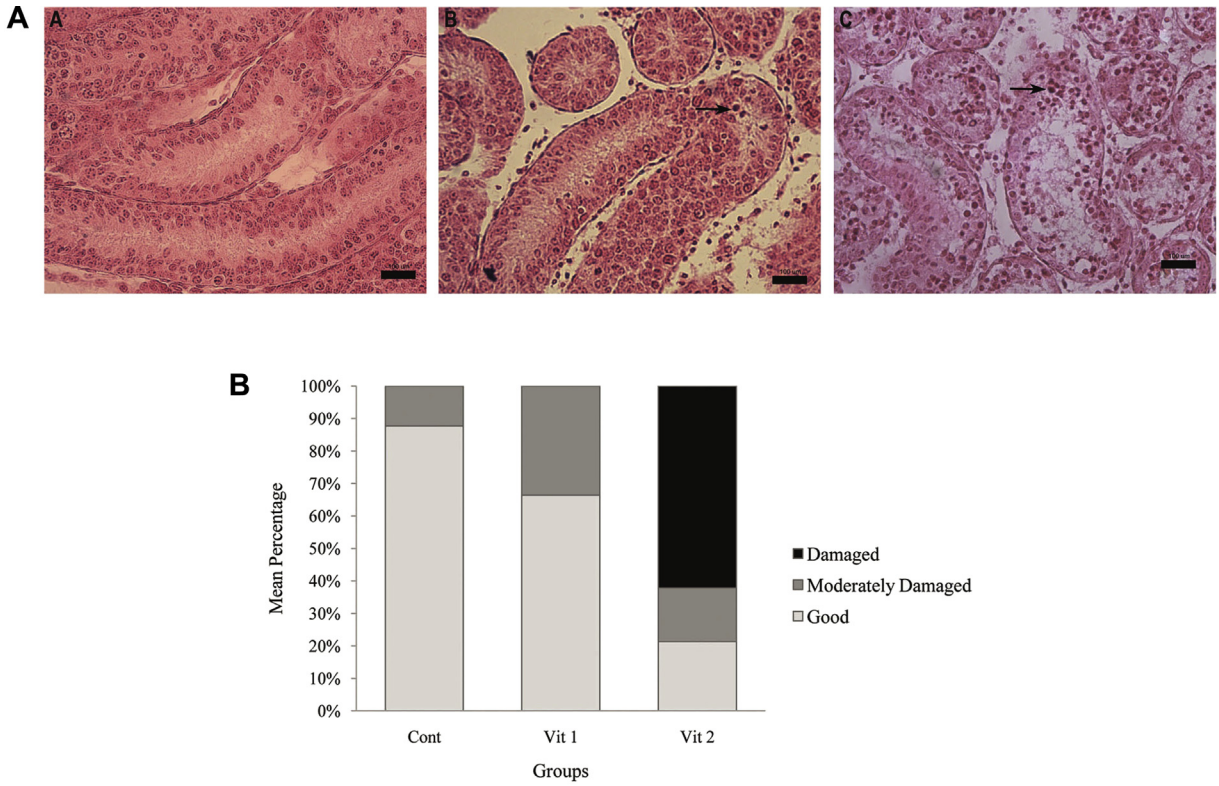

Fig. 3. (A) Hematoxylin-eosin staining of cultured testis fragments at 3 hours of culture in all experimental groups. (A, B, C) Histologic sections of fresh control (Cont) and vitrified-warmed testis fragments in vitrification 1 (Vit 1) and vitrification 2 (Vit 2) groups, respectively. Black arrow: pyknotic nuclei, original magnification ×400. (B) Semiquantitative data (good, moderately damaged, and damaged seminiferous tubules) of cultured testis fragment at 3 hours of culture in all experimental groups.

culture, and despite the nonsignificant decrease in Vit 2 group, it remained constant in all groups up to 20 hours of culture compared to 3 hours of culture (Fig. 5).

3.3.5. Fas

A: Vit 1, Vit 2, and control groups showed the highest expression of *Fas* at 0, 3, and 20 hours of culture, respectively.

B: Although relative expression of *Fas* increased significantly in both vitrification groups up to 3 hours of culture, it was reduced significantly until 20 hours of culture (Fig. 5).

3.3.6. Fas ligand

A: Highest expressions of *Fas ligand* at 0, 3, and 20 hours of culture were observed in Vit 2, Vit 1, and both vitrification groups, respectively.

B: Although relative expression of *Fas ligand* increased significantly in all groups up to 3 hours of culture, it decreased in control and Vit 1 group and increased significantly in Vit 2 group at 20 hours of culture. Expression of *Fas ligand* in all experimental groups was significantly higher at 20 hours than 0 hours of culture (Fig. 5).

3.3.7. p53

A: Relative expression of *p53* was significantly higher in control and Vit 2 groups at 0 and 20 hours of culture than Vit 1 group.

B: Although relative expression of *p53* increased nonsignificantly in Vit 2 group up to 20 hours of culture, it declined significantly in Vit 1 group (Fig. 5).

4. Discussion

In recent years, testis vitrification has been introduced as an advantageous technique for fertility preservation in boys suffering from cancer [4,8,17–19]. Because of serious problems of testis grafting which include inadequate oxygen and nutrient supplies [20], ischemic stress [21], and contamination by malignant cells [22], IVC of testicular fragments has been considered to be an applicable technique. In 2011, fertile offspring were obtained by microinjection of sperm and round spermatid that developed from cultured testicular fragments [15]. They reported the birth of mice after culture of cryopreserved testicular tissue 2 years later [19]. According to their results and despite their success, degeneration of testicular tissue after cryopreservation and culture has been non-negligible. Hence, an understanding of involved molecular mechanisms seems to be essential for improvements of cryopreservation and culture techniques. In this study, we have attempted to understand the molecular reasons of cell death after vitrification of testicular tissue and also after IVC for 20 hours.

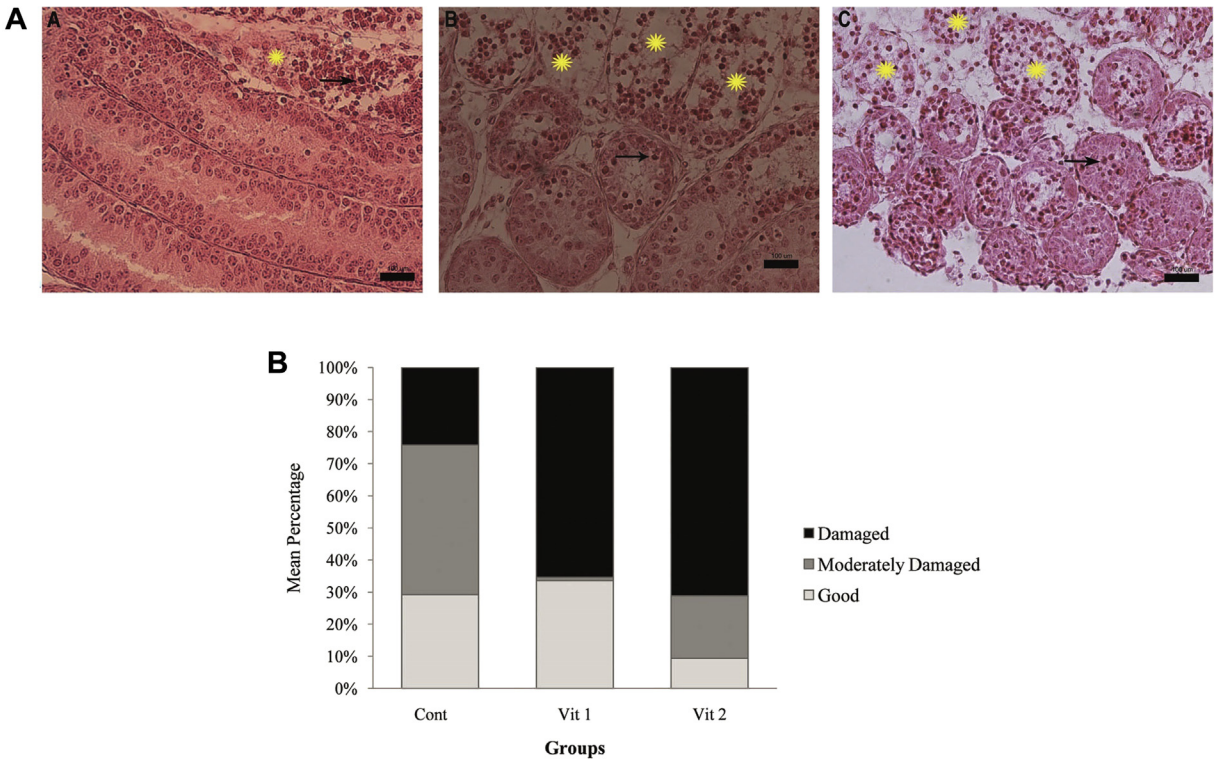

Fig. 4. (A) Hematoxylin-eosin staining of cultured testis fragments at 20 hours of culture in all experimental groups. (A, B, C) Histologic sections of fresh control (Cont) and vitrified-warmed testis fragments in vitrification 1 (Vit 1) and vitrification 2 (Vit 2) groups, respectively. Black arrow: pyknotic nuclei, yellow star: degenerated central tubules. Original magnification ×400. (B) Semiquantitative data (good, moderately damaged, and damaged seminiferous tubules) of cultured testis fragment at 20 hours of culture in all experimental groups. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

In accordance with the study by Curaba et al. [8], testis integrity was well preserved in Vit 1 protocol but contrasting with the study by Gouk et al. [5] using EG as the only permeable cryoprotectant in vitrification solution (Vit 2 group) was not sufficient for preservation of testis tissue integrity. Therefore, it appears that use of EG and DMSO combination would be better for preservation of the integrity of testicular tissue.

Obvious tissue alterations at the end of the culture period in control and both vitrification groups indicate the deficiency of the culture technique. Existence of intact tubules in the marginal zone of cultured tissue could indicate supportive ability of the culture condition.

Low temperature exposure, vitrification, and warming procedure stresses may be the main reasons for significant increase of early apoptosis in both vitrification groups. Despite the success of Vit 1 group in tissue integrity preservation, it showed the highest rate of early apoptosis during the first hours of the culture period. This could be attributed to DMSO toxicity [23]. In both vitrification

Table 1
Mean percentage of viability, early apoptosis, late apoptosis, and necrosis in control (Cont), vitrification 1 (Vit 1), and vitrification 2 (Vit 2) groups.

Cell population	Group	0-h Culture	3-h Culture	20-h Culture
Living cells	Cont	$75.6 \pm 1.65^{A,a}$	$72.40 \pm 0.98^{A,b}$	$46.82 \pm 1.76^{A,c}$
	Vit 1	$40.62 \pm 0.89^{B,a}$	$26.14 \pm 1.78^{B,b}$	$11.83 \pm 1.27^{B,c}$
	Vit 2	$29.65 \pm 0.38^{C,a}$	$18.25 \pm 1.26^{C,b}$	$11.10 \pm 0.74^{B,c}$
Early apoptosis	Cont	$4.65 \pm 0.37^{A,a}$	8.40 ± 1.15^{b}	$6.61 \pm 0.22^{A,b}$
	Vit 1	$37.34 \pm 0.91^{B,a}$	6.09 ± 0.43^{b}	$1.46 \pm 0.28^{B,c}$
	Vit 2	$30.72 \pm 2.2^{C,a}$	9.07 ± 1.23^{b}	$0.76 \pm 0.11^{B,c}$
Late apoptosis	Cont	$8.25 \pm 0.4^{A,a}$	$11.76 \pm 0.48^{A,b}$	$31.86 \pm 1.08^{A,c}$
	Vit 1	$14.46 \pm 0.86^{B,a}$	$28.64 \pm 1.9^{B,b}$	$37.18 \pm 2.34^{A,c}$
	Vit 2	$24.83 \pm 1.07^{C,a}$	$51.17 \pm 1.45^{C,b}$	$24.71 \pm 1.36^{B,a}$
Necrosis	Cont	$11.72 \pm 1.82^{A,a}$	$7.48 \pm 1.41^{A,a}$	$14.70 \pm 1.74^{A,b}$
	Vit 1	$7.57 \pm 0.74^{B,a}$	$39.07 \pm 3.06^{B,b}$	$50.11 \pm 3.62^{B,c}$
	Vit 2	$14.78 \pm 2.37^{A,a}$	$21.45 \pm 2.07^{C,a}$	$63.86 \pm 1.33^{C,b}$

Data were expressed as mean percentage ± SEM. Significant differences were considered as $P < 0.05$. (A vs. B, C) and (B vs. C) Significant differences in each time of culture between groups ($P < 0.05$). (a vs. b, c) and (b vs. c) Significant differences in each group during culture period ($P < 0.05$).

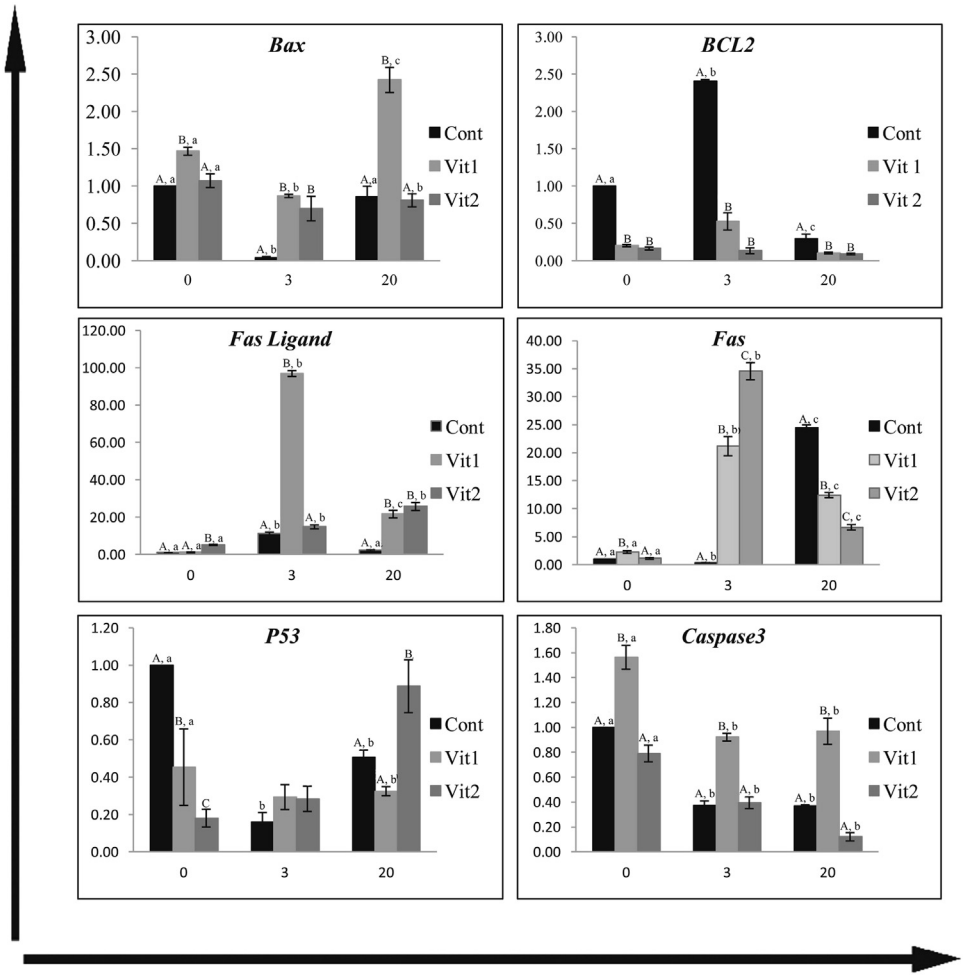

Fig. 5. Relative gene expression of *Bax, Bcl2, Fas Ligand, Fas, P53,* and *Caspase 3* in control (Cont), vitrification 1 (Vit 1), and vitrification 2 (Vit 2) groups. Data were expressed as mean percentage ± SEM. (A vs. B, C) and (B vs. C) Significant differences in each time of culture between groups ($P < 0.05$). (a vs. b, c) and (b vs. c) Significant differences in each group during culture period ($P < 0.05$).

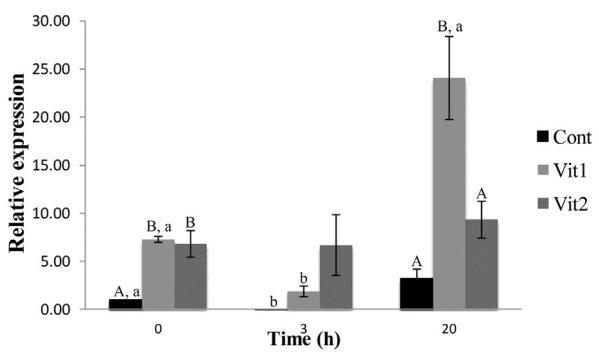

Fig. 6. Ratio of *BAX* to *BCL2* relative expression in control (Cont), vitrification 1 (Vit 1), and vitrification 2 (Vit 2) groups. Data were expressed as mean percentage ± SEM. (A vs. B) Significant differences in each time of culture between groups ($P < 0.05$). (a vs. b) Significant differences in each group during culture period ($P < 0.05$).

groups, induction of late apoptosis and reduction of early apoptosis until 3 hours of culture could signify the completion of cell apoptosis which started at 0 hours of culture.

Necrosis is another damaging effect of cryopreservation [14]. In the present study, higher percentage of necrotic cells in vitrification groups than control can confirm the destructive effects of the vitrification protocols which are then augmented by the culture protocols. Increase of cell death by apoptosis and necrosis can be linked to the destructive alterations of testicular fragments at the end of the culture period in all experimental groups. This can be explained by the deficiency of culture technique. Higher percentage of cell death in vitrification groups than control group indicates that cell death is induced by vitrification and warming procedure in addition to the culture procedure.

Bcl2 super family is a group of regulatory proteins, which is involved in apoptosis incidence. This family consists of proapoptotic proteins (such as *Bax, Bad*) that accumulate in the outer membrane of mitochondria and

interact with antiapoptotic proteins (such as *Bcl2*). This results in the release of cytochrome c and leads to apoptosis by intrinsic pathway [24]. Accumulation of spermatogonia and spermatocyte in testis of transgenic mice with *Bax* "knock out" or *BCL2* over expression leads to infertility [25,26], also involving apoptosis intrinsic pathways and testicular cell death. Increase of *BAX* and decrease of *BCL2* expression at all evaluation times of culture in vitrification groups compared to the control could indicate the involvement of intrinsic pathway in apoptosis induction after vitrification and culture. *Bcl2* increase, *Bax* decrease, and more apoptosis induction up to 3 hours of culture in control group could explain the involvement of another pathway (extrinsic pathway) in cell apoptosis.

Genetic disruption of proapoptotic and antiapoptotic protein balance could destroy the normal development of spermatogenesis [27]. During the culture process, ratio of *BAX* to *Bcl2* (apoptotic index) was higher in vitrification groups than control group, which could be a reason for more apoptotic induction in those groups. Higher increase of this index in Vit 1 group could be triggered by the existence of DMSO.

Activation of *p53* is followed by upregulation of proapoptotic proteins such as *Bax* and death receptor proteins [28,29]. Based on the study by Maheshwari et al. [30], testis treatment by H_2O_2 causes concurrent increase of *p53* and *BAX* expression. In our study, lack of coordination between *p53* and *BAX* expression after vitrification and culture process could show independence of intrinsic pathway from *p53* expression.

Fas and *Fas ligand* are two apoptotic genes that play an important role in triggering testis extrinsic apoptosis pathway [31]. In vitrification groups, concordance increment of *Fas* and *Fas ligand* and late apoptotic cells during culture could show the involvement of extrinsic pathway in cell death. Unexplained upregulation of *Fas ligand* mRNA expression after germ cells treatment by H_2O_2 was seen in the study by Maheshwari et al. [30]. We also observed a sudden unexpected upregulation of *Fas ligand* expression at 3 hours of culture in Vit 1 group. This might show the toxicity effect of vitrification solutions.

H_2O_2 treatment of rat testicular germ cells and human spermatozoa [30,32] caused increase of *Caspase 3* activity. Involvement of *Caspase 3*, *8*, and *9* in the first spermatogenesis wave of rat was reported by Moreno et al. [33]. In our study, although *Caspase 3* decreased during culture period in all groups, apoptosis increased at the end of the culture period. Cells may be thought to utilize *Caspase 3* that exists normally in the cytosol [34]. At the end of the culture, higher expression of *Caspase 3* in Vit 1 group compared to the other groups was concurrent with higher apoptosis incidence in this group. In addition, higher necrosis rate in Vit 2 group than in other groups at the end of the culture period could trigger other molecular mechanisms that are not evaluated in this study.

4.1. Conclusion

Although existence of DMSO in vitrification solution 1 could improve integrity preservation of testis tissue, it induces significant apoptosis during the culture period. It seems that Vit 1 group in comparison with Vit 2 group induces cell death more via apoptotic pathway than necrosis and also apoptosis incidence after vitrification may occur independent of *p53*.

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Competing interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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