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Original Article

Assessment of Apoptosis-Related Genes in Autografted/ Vitrified Ovarian Tissue in Mice

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Abstract

Background: One of the new emerging techniques to preserve reproductive potential of cancer patients is cryopreservation of ovarian fragments prior to medical treatment and their re-transplantation after healing. In order to investigate and compare apoptosis in ovarian tissue, the current study has done while using different vitrification solutions.

Methods: The ovaries of Balb/c mice were removed from the body. R ovaries autotransplanted and L ovaries vitrified and thawed in different solutions. Apoptosis-related genes (Bax, Bcl-2, Bid, P53) assessed by RT-PCR.

Results: The results have shown that Bax and P53 expression showed significant changes in DCV3, but in DCV2 only p53 expression decreased considerably in vitrified-thawed group.

Conclusion: We concluded that cryoprotectant solution is important for preserving tissue in vitrification process and in our study DCV2 showed better results than DCV3.

Introduction

The incidence of cancers has dramatically increased around the world.[1] Advances in cancer treatment have greatly enhanced long-term survival and quality of life but the side effects of cytotoxic anticancer treatments frequently damage ovarian tissue due to the susceptibility of the ovaries to ROS production.[2] Ovarian tissue cryopreservation and transplantation are the most emerging strategy to preserve fertility in these patients.[3,4] It is the only option that can be offered to prepubescent girls and implemented without any delay in treatment.[5-7]

In vitro maturation of follicles and ovarian tissue transplantation are two methods for obtaining mature oocytes from frozen-thawed ovaries. So far, ovarian cryopreservation and transplantation procedures have been almost exclusively limited to avascular cortical fragments in both experimental and clinical studies.[8-10] The biophysical basis of cryo-damage in cells and tissues is not clear yet.

Autotransplantation of frozen-thawed ovarian tissue has used for restoration of endocrine function numerously in various species.[11-13] Recently live births have achieved through auto-transplantation of cryopreserved ovarian tissue.[12] However, after transplantation of vitrified–warmed mouse ovarian tissue, the pre-antral and antral follicles have lost but the primordial and primary follicles were intact.[14,15] Apoptosis is a process of biologically meaningful and the underlying mechanism of ovarian follicle degeneration during atresia.[16,17] There are at least two pathways that lead to ovarian tissue apoptosis: extrinsic pathway such as Fas/FasL system[18,19] and intrinsic pathway that involved several apoptotic genes, such as Bcl-2, Bax, p53, and BIRC5.[20-22] Bcl-2 is expressed in granulosa cells of both fetal and adult ovaries and p53 protein is expressed in the apoptotic granulosa cells of atretic follicles. Moreover, p53 can induce apoptosis by the activation of Bax gene, which encodes an apoptosis-inducing factor.[23,24] Apoptosis also plays an important role in cryo-injuries, mainly via activation caspases and the Fas system.[25]

One of the main causes of follicle loss after transplantation is the ischemia and lack of adequate blood supply[26-28] that is associated with higher level of apoptotic genes expression such as Bcl-2 and p53.[29,30]

For this reason, researchers transfer tissues to rich vascularization site, such as a kidney capsule. Liu et al indicated that transplantation after cryopreservation caused major loss of primordial follicles in the transplanted ovaries in newborn mice.[31]

It is expected that different cryopreservation protocols (slow conventional freezing or vitrification) and usage of different cryoprotective agent (CPA) type will results in different effects on both cellular integrity and tissue functions including apoptosis.[32] The types of CPA commonly used in ovarian tissue vitrification are dimethyl sulfoxide (DMSO), propanediol (PrOH) and ethylene glycol (EG) as permeating CPAs, and sucrose, trehalose and Ficoll as non-permeating CPAs. According to

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several recent studies, using two different forms of CPAs together is less harmful and more productive than using just one type of CPA alone.[33,34] however, the efficacy of CPA compositions and concentrations for ovarian tissue vitrification are not yet well documented.

Therefore, establishing the optimal vitrification protocol is crucial, with consideration of the best vitrification process, taking into account the CPA type, concentration, and equilibration period. Various CPA compositions have been studied in an effort to determine the best cryopreservation procedure for ovarian transplantation (OT).[35,36]

The aim of the current study is to investigate the influence of different vitrification protocol on the development of apoptosis in cryopreserved/ auto-transplanted ovaries in mice.

Material and methods

Ethical issues

All phases of current experiment were in accordance to published guideline of "the Care and Use of Laboratory Animals" (NIH Publication No. 85-23, revised 1996) and approved by the local ethical committee of Tabriz University of Medical Sciences.

Animals and ovarian tissue preparation

All chemicals and media used in the current experiment were purchased from Sigma-Aldrich Company (Germany) unless otherwise mentioned. Six-week-old female Balb/c mice (n = 20) were accommodated in controlled condition with 12-hour light/12-h dark, 22-25 °C and 55% humidity. The mice had enough access to water and food. To investigate the distinct effects of different direct cover vitrification (DCV) on stability and successful transplantation of ovaries in mice model, animals were anesthetized by an intraperitoneal injection of 75 mg/kg ketamine. Thereafter, both left and right ovaries from each mouse were dissected and surrounding fat and mesentery separated carefully. For control comparison of ovary transplantation with our freezing method, the right ovaries were submerged and washed in α-MEM supplemented with 20% fetal bovine serum (FBS, Gibco) for 2 minutes and autologously transplanted into subcutaneous tissue and left ovaries after vitrification/ thawing was autotransplanted subcutaneously.

Vitrification

We followed vitrification and thawing protocol as previously by Chen et al. Left ovaries from each mouse were randomly allocated to two main vitrification groups. Both cryo-protectant and warming solutions were prepared in Dulbecco's phosphate-buffered saline (DPBS). In current experiment, cryoprotectants solutions in the equilibration and vitrification solutions was formulated as follows: 5% EG + 5% DMSO + 0.5 M sucrose + 20% FBS (DCV1); 10% EG + 10% DMSO + 0.5 M sucrose + 20% FBS (DCV2) and 15% EG + 15% DMSO + 0.5 M sucrose + 20% FBS (DCV3). After washing in α-MEM supplemented with 20% FBS for 2 minutes, ovaries from group DCV2 (n = 10) were equilibrated sequentially in DCV1, 2 and vitrified in DCV 2, while in ovaries from group DCV3 (n = 10) equilibrated in three equilibration solutions DCV1, 2 and 3, and vitrified in DCV3. The ovarian tissues were pretreated with equilibration and vitrification solutions for 10 and 2 minutes in room temperature, respectively. Ovarian tissue was put in plastic cryotube and liquid nitrogen was directly applied onto the vitrification solution (DCV). The cryotube was placed into a liquid nitrogen tank for 10 minutes.

Thawing

Following experimental procedure of freezing, the vitrified ovaries were warmed in room temperature for 10 seconds and then into a 38 °C water bath for 20 seconds, so that ice melted. The ovarian tissue of cryotubes was suspended in 1 mL of serially descending concentrations of sucrose (1, 0.5 and 0.25 M) and DPBS at room temperature for 10 minutes. Warmed ovaries were equilibrated in α-MEM medium supplemented with 20% FBS for 30 minutes for following procedure. To evaluate the possible toxicity of vitrification and thawing procedure, the ovaries were exposed to the cryoprotectant solution and passed through all stages of vitrification and warming procedure except plunging in liquid nitrogen as previously described.

RNA extraction and first-strand cDNA synthesis

For all autotransplanted animals, the ovary was removed for RNA extraction under standard sterile surgical method. Total RNA was extracted from ovary tissue using Trizol Reagent (Invitrogen, USA) according to the manufacturer's description and treated with RNase-free DNase to remove any residual genomic DNA. Single stranded cDNAs were synthesized by incubating total RNA [1 μg] with RevertAid H Minus M-MuL V Reverse transcriptase [200 U], oligo-[dT]18 primer [5 μM], Random Hexamer Primer [5 μM], dNTPs [1 mM], and RiboLock RNase-inhibitor [20 U], for 5min at 25°C followed by 60 minutes at 42 °C in a final volume of 20 μL. The reaction was terminated by heating at 70 °C for 5 minutes.

Real-time relative quantitative RT-PCR

Quantitative real time PCR was done using the Corbett Life Science (Rotor-Gene 6000) System is using 2 μL of an 8-fold diluted cDNA in each PCR reaction in a final volume of 20 μL. Each PCR reaction contained 150 nM of primers and 1 × FastStart SYBR Green Master (Roche). Sequences of primers and Annealing temperature are listed in Table 1. PCR amplifications were performed by the following three cycle programs: (1) denaturation of cDNA [1 cycle: 95°C for 10 minutes]; (2) amplification [40 cycles: 95 °C for 15 seconds, annealing temperature °C for 30 seconds 63°C for 34 seconds]; (3) melting curve analysis [1 cycle: 65 to 95 °C with temperature transition

rate 1 °C/s]. β-actin [Actb] mRNA expression levels were used to calculate relative expression levels. The relative quantification was performed by by 2[−ΔΔCt]. The specificity of the PCR reactions was verified by generation of a melting curve analysis followed by gel electrophoresis, visualized by ethidium bromide staining.

Standard curve

Efficiency of RT-PCR reaction was determined by standard curve, which was derived from the 10-fold serial dilution of a positive PCR product by a customary RT-PCR. Logarithms of concentrations were plotted against target gene cycling threshold (Ct) of serial dilution. Bid, P53, Bcl-2, Bax and β-actin efficiencies were 89%, 95%, 95%, 100% and 100% respectively.

Statistical analysis

Normal distribution of data was evaluated using Stata software with qnorm program version 11. Data was analyzed by statistical SPSS software, version; 27.0.1. Variables that had normal distribution were reported as means and standard deviations. Medians were reported for the variables whose distribution deviated from the normal distribution. Differences between groups were evaluated using the independent samples *t* test and Mann–Whitney test and comparisons gene expression levels inter group (frozen-thawed ovarian tissue and the grafts) was performed with the paired-samples *t* test and Wilcoxon test. All tests were two-tailed and a 5% significance level was applied.

Results

Assessment of Bax, Bcl-2, P53 and Bid expression in the auto-transplanted ovaries vitrified in DCV2 solutions

The median expression of P53 mRNA in frozen-thawed ovarian tissue of the DCV2 solution were decreased about 7-fold compared to controls ($P = 0.002$; Figure 1).

Also, the expression of Bax mRNA in DCV2 were decreased compared to controls. However, this decrement not statistically significant ($P = 0.095$; Figure 2).

On contrary, the Bid expression, not only decreased in DCV2 grafts, but also the transcript levels increase, although no significant difference was found regarding parallel control ($P = 0.099$; Figure 3).

Furthermore, there were no significant differences in median Bcl-2 mRNA expression between examine groups ($P = 0.506$; Figure 4).

Bax mRNA expression was positively correlated with the expression of P53 transcripts in DCV2 and controls (rs = 0.800, $P = 0.005$, rs = 0.643, $P = 0.013$ respectively). Also, Bax mRNA expression was positively correlated with the expression of Bid transcripts in DCV2 solution (rs = 0.900, $P < 0.001$).

Assessment of Bax, Bcl-2, P53 and Bid expression in the auto-transplanted ovaries vitrified in DCV3 solutions

In frozen-thawed ovarian tissue placed in a DCV3 solution, median expression of Bax and P53 mRNA were increased about 12-fold and 3-fold compared to controls ($P = 0.005$ and $P = 0.012$ respectively) (Figures 5 and 6). Also, the median expression of Bid mRNA in DCV3 solution was increased compared to controls. However,

Table 1. Primer's list

Gene	Forward primer	Reverse primer	Annealing temperature°
Bax	TGAAGACAGGGGCCTTTTTG	AATTCGCCGGAGACACTCG	57 °C
Bcl-2	ATGCCTTTGTGGAACTATATGGC	GGTATGCACCCAGAGTGATGC	57 °C
P53	ACTCAGACTGACTGCCTCTG	TCTCAGCCCTGAAGTCATAA	57 °C
Bid	AGCAAATGTTCCCTCCGCTTCTGT	GTAGGCTGTGGCGGCTCGTG	60 °C
Actb	GGGCACAGTGTGGGTGAC	CTGGCACCACACCTTCTAC	57 °C

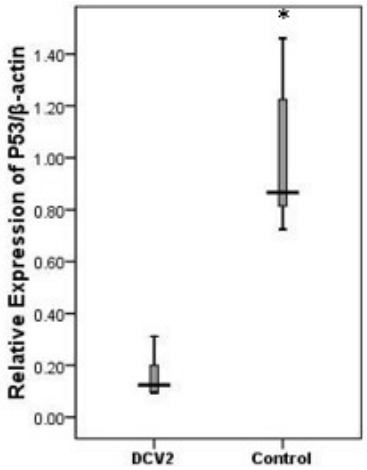

Figure 1. The median expression of P53 in frozen-thawed ovarian tissue of the DCV2 solution were decreased compared to controls *$P < 0.05$

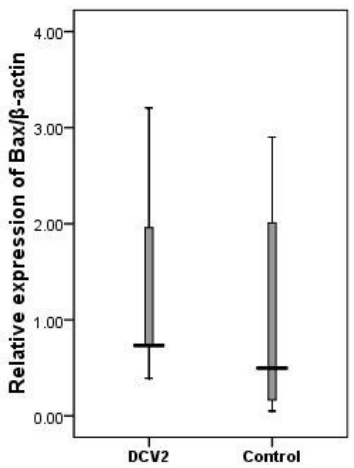

Figure 2. Expression of Bax in DCV2 solution were not statistically significant compared to controls

this increment was not statistically significant ($P = 0.085$; Figure 7).

However, there were no significant differences in Bcl-2 mRNA expression between examine groups ($P = 0.325$; Figure 8).

Bax mRNA expression was positively correlated with the expression of P53 and Bid transcripts in DCV3 and controls (rs = 0.996, $P < 0.001$, rs = 0.994, $P < 0.001$ and rs = 0.929, $P < 0.001$, rs = 0.886, p < 0.001, respectively).

Discussion

Cryopreservation and transplantation of ovarian tissue has a long history in animal and early human studies. Vitrification is a rapid and simple method which has been applied for cryopreservation of ovarian tissues due to its minimal changes in morphology and ultrastructure.[37,38] One of the difficulties of vitrification method is the use of high concentrations of toxic cryoprotectants that can be harmful to the cells and tissues.[34] Therefore, for the prevention of damage due to osmotic pressure, increasing the rate of water replacement with cryoprotectants is necessary.[39]

Ovarian tissue transplantation can be helped to preserve fertility and ovarian function in women experiencing premature ovarian failure and infertility specially for those who survived from cancer treatments.[4]

The first report from human live birth after transplantation of cryopreserved ovarian tissue was in a patient who had undergone ovarian tissue cryopreservation before chemotherapy for Hodgkin's disease.[40] However, follicular damage due to ischemia after transplantation significantly shortens the functional life span of a graft.[41]

Ischemia injury is associated with apoptosis incidence in several organs and survival rate.[29,42] In current study, we hypothesized that ischemia of the ovarian tissues can alter expression of p53 and Bcl-2. In this regard we evaluate the effect of different concentrations of cryoprotectant solutions on the expression of Bax, Bcl-2, P53 and Bid expression on fresh and cryopreserved-thawed ovarian tissue.

Apoptosis can be started by the extrinsic or intrinsic pathways. Bcl-2 and Bax are expressed in oocytes, granulosa cells, and stroma of both fresh and warmed-vitrified ovaries. However, vitrification doesn't increase apoptosis via the intrinsic pathway in follicles.[34]

The expression of Bax and Bcl-2 were evaluated because vitrification has been reported to initiate the intrinsic apoptosis pathway.[43-45] The p53 protein is an anti-proliferative transcription factor that controls genomic

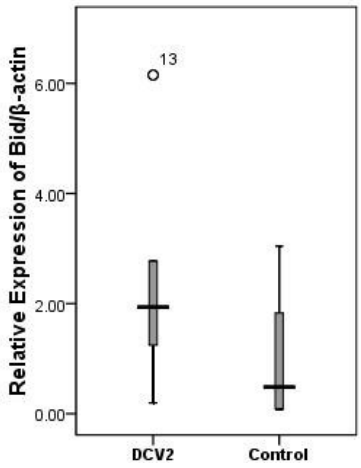

Figure 3. The Bid expression were not significantly decreased in DCV2 grafts compared to control group

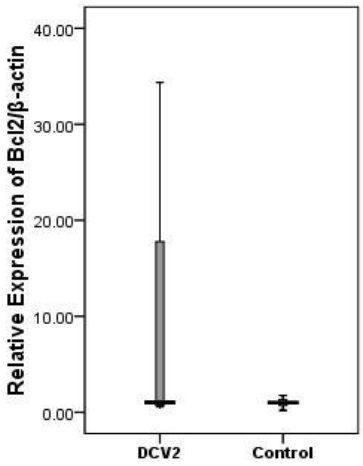

Figure 4. Median Bcl-2 expression in DCV2 solution had no significant differences in groups

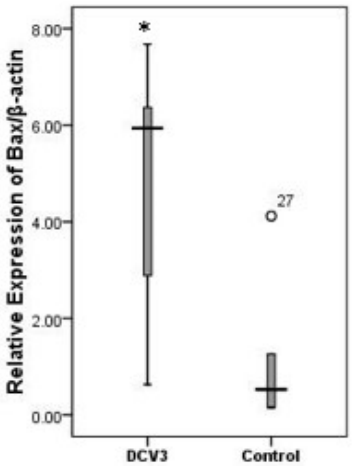

Figure 5. Median expression of P53 in DCV3 solution was increased significantly compared to controls (*$P < 0.05$)

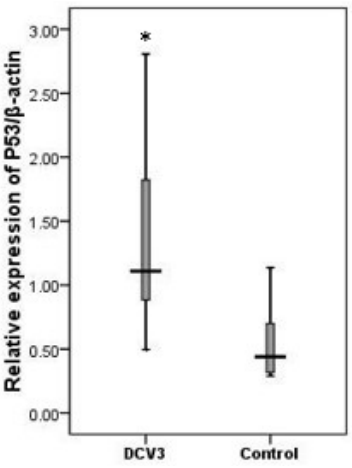

Figure 6. Median expression of Bax in DCV3 solution were increased compared to controls (*$P < 0.05$)

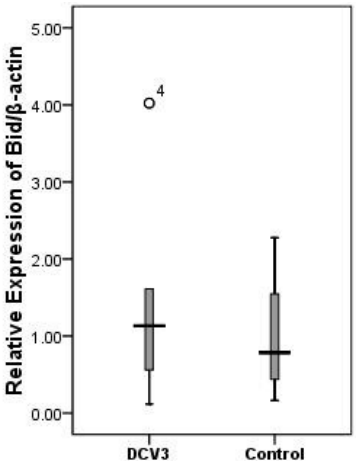

Figure 7. Median expression of Bid in DCV3 solution was not significantly increased compared to controls

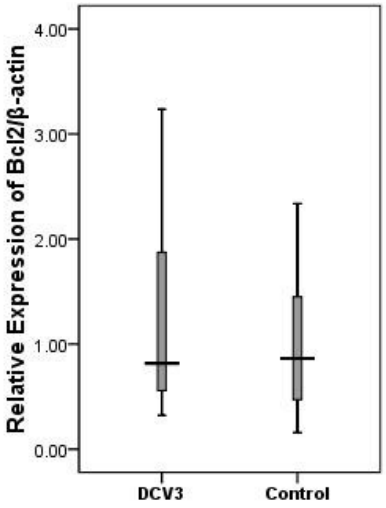

Figure 8. Bcl-2 expression in DCV3 solution has no significant differences between examine groups

integrity by inducing cell cycle arrest or apoptosis. This protein is expressed in the apoptotic granulosa cells of atretic follicles.[46]

Studies have shown that apoptosis rate increased after transplantation of ovarian tissue. In line with this, Damásio et al reported that Bax expression was higher in fresh/vitrified transplanted groups compared to control group.[47]

On the contrary, Abdollahi et al stated that the level of FasL, Bcl-2, Bax, p53, and caspase3 mRNA and Bax/Bcl-2 ratio was similar in non-vitrified and vitrified groups.[48] Jafarabadi et al also reported that the expression of some pro and anti-apoptotic genes in vitrified-thawed ovarian tissues were not changed compared to non-vitrified ones, but the expression of Fas and caspase8 was increased in this group.[49]

Our data indicated that the expression of some pro-apoptotic genes, including Bax, and Bid and anti-apoptotic gene Bcl-2 have no significant differences between control group and DCV2. But Bax expression increased notably in DCV3 compared to control group.

In the current study, p53 expression showed considerably changes between groups in two different concentrations of vitrified solution (DCV2 and DCV3).

Conclusion

We concluded that concentration of cryoprotectant solutions is important in ovarian tissue preservation in vitrification process before transplanting. while low concentration of vitrification solution may have less bad effects on tissue survival.

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Author Contribution

Conceptualization: Ali Abedelahi.
Investigation: Melika Izadpanah, Mohammad Reza Aliparasti.
Methodology: Ali Abedelahi.
Software: Melika Izadpanah, Mohammad Reza Aliparasti.
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Validation: Melika Izadpanah, Mohammad Reza Aliparasti.
Visualization: Melika Izadpanah, Mohammad Reza Aliparasti.
Writing–original draft: Melika Izadpanah, Mohammad Reza Aliparasti.
Writing–review & editing: Melika Izadpanah, Mohammad Reza Aliparasti.

Competing Interests

There is no competing interest to this study.

Ethical Approval

All steps of experimental protocols were confirmed by the local ethics committee of Tabriz University of Medical Sciences and animals were treated under the previously published guidelines (NIH, 1986).

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