



# Effect of selenium on freezing-thawing damage of mice spermatogonial stem cell: a model to preserve fertility in childhood cancers

Mandana Beigi Boroujeni<sup>1,2</sup>, Fatemeh Peidayesh<sup>3</sup>, Afshin Pirnia<sup>1</sup>, Nasim Beigi Boroujeni<sup>1</sup>, Seyyed Amir Yasin Ahmadi<sup>4</sup>, Mohammadreza Gholami<sup>5</sup>

<sup>1</sup>Razi Herbal Medicines Research Center, <sup>2</sup>Department of Anatomical Sciences, <sup>3</sup>Student Research Committee, Lorestan University of Medical Sciences, Khorramabad, Iran; <sup>4</sup>Pediatric Growth and Development Research Center, Institute of Endocrinology and Metabolism, Iran University of Medical Sciences, Tehran, Iran; <sup>5</sup>Department of Anatomical Sciences, Kermanshah University of Medical Sciences, Kermanshah, Iran

**Contributions:** (I) Conception and design: MB Boroujeni; (II) Administrative support: M Gholami, MB Boroujeni; (III) Provision of study materials or patients: M Gholami, MB Boroujeni; (IV) Collection and assembly of data: F Peidayesh, A Pirnia, NB Boroujeni; (V) Data analysis and interpretation: F Peidayesh, A Pirnia, NB Boroujeni, SA Ahmadi; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

**Correspondence to:** Mohammadreza Gholami. Department of Anatomical Sciences, Kermanshah University of Medical Sciences, Kermanshah, Iran. Email: rezagholami57@gmail.com.

**Background:** During treatment of childhood cancers, fertility of boys may be affected. Therefore, freezing spermatogonial stem cell (SSC) is recommended. However, freezing-thawing process may cause damage to SSCs. This study was conducted to evaluate protective effects of selenium on freezing-thawing damage of mice SSCs using investigation of cell viability and investigation of apoptosis related genes expression including *Fas*, *Caspase3*, *Bcl2*, *Bax* and *P53*.

**Methods:** SSCs were extracted from 80 6-day-old mice. The SSCs were divided into four groups: cryopreservation along with selenium (low and high dose), vitrification along with selenium (low and high dose), cryopreservation control, and vitrification control. Trypan blue staining and real-time polymerase chain reaction (real-time PCR) were used to investigate cell viability and gene expression, respectively.

**Result:** Comparison of cell viability in the experimental groups did not show a significant association. Expression of *Fas* and *Caspase3* was significantly lower in cryopreservation group with low-dose selenium. Expression of *Bcl2* was significantly lower in cryopreservation group with high-dose selenium. Expression of *Bax* and *Caspase3* was significantly lower in vitrification group with low-dose selenium, and expression of *P53* was significantly upper. Expression of *Bax* and *Fas* was significantly lower in vitrification group with high-dose selenium, and expression of *P53* was significantly upper ( $P < 0.001$ ).

**Conclusions:** Selenium had dose dependent effect on apoptosis related genes profile. The only evident effect was the effect of low-dose selenium in cryopreservation on inhibition of apoptosis via extrinsic pathway.

**Keywords:** Selenium; cryopreservation; apoptosis; fertility preservation; cancer

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## Introduction

### Background

In mankind the origin of germ cells is primordial germ cells (PGC) in both genders (1). PGCs are developed by bone morphogenic protein (BMP) signaling (such as BMP-4) (2). In male PGCs are surrounded by Sertoli cells in genital crest and seminiferous cords are created. Then PGCs are converted to gonocytes. These cells proliferate until arrest phase. After birth (in mice), they migrate lumen toward basal membrane of seminiferous cords and create spermatogonium-A and spermatogonium-B, and then back toward the lumen. One level of spermatogonium-A formation is called spermatogonial stem cell (SSC). These stem cell have self-renewal potency and therefore they can be stored for fertility preservation in future (3).

Infertility is a challenge of nowadays medicine. Male related infertilities are investigated using sperm analysis due to availability of sperm; but human oocytes are not easily available. Many sperm studies have shown that chemotherapy and radiotherapy can affect quality of sperm both in testicular cancers (4) and in other cancers (5). For instance, acute lymphoblastic leukemia (ALL) is common cancer in childhood. Poganitsch-Korhonen *et al.* mentioned that spermatogonial quantity decreased in treatment of ALL, based on biopsy studies (6). Therefore, a solution for fertility preservation in such conditions is storing frozen SSCs in prepubertal boys. Since at such ages puberty has not occurred, there is no sperm for preservation. In addition, SSCs have further potencies. Therefore, in such cases SSCs should be regarded for freezing instead of sperms. The process of freezing can be slow (cryopreservation) or rapid (vitrification) (3,7).

Many *in vitro* and *in vivo* studies have done both for animal modeling of testicular damage and for effects of different interventions in such conditions. These models are based on induction of cytotoxicity, oxidative stress [induction of reactive oxygen species (ROS)] (8) as well as induction of apoptosis (9). For example, for *in vivo* models, testicular torsion-detorsion for induction of ROS (10,11), gentamycin toxicity (12), or cisplatin induced testicular damage (13), and for *in vitro* models freezing-thawing damage (8) can be mentioned. The interventions include melatonin (14), ghrelin (15), selenium (16), medical plants (17) and so on.

### Rationale

Childhood cancers requires chemotherapy. During cancer treatment, fertility of boys may be affected. Therefore, freezing SSC was recommended. However, freezing-thawing process may cause damage to SSCs. Hence adjuvant interventions should be carried out to reduce this damage. Investigation of this evidence gap requires animal model.

### Objectives

Due to the importance of reduction of freezing-thawing damage, this study is conducted to evaluate protective effects of selenium on freezing-thawing damage of immature mice SSCs using investigation of cell viability and investigation of apoptosis related genes expression. These genes were *Fas*, *Caspase3*, *Bcl2*, *Bax* and *P53*. The practical aim of this study was to design an animal model of fertility preservation in order to use human research in childhood cancers.

## Methods

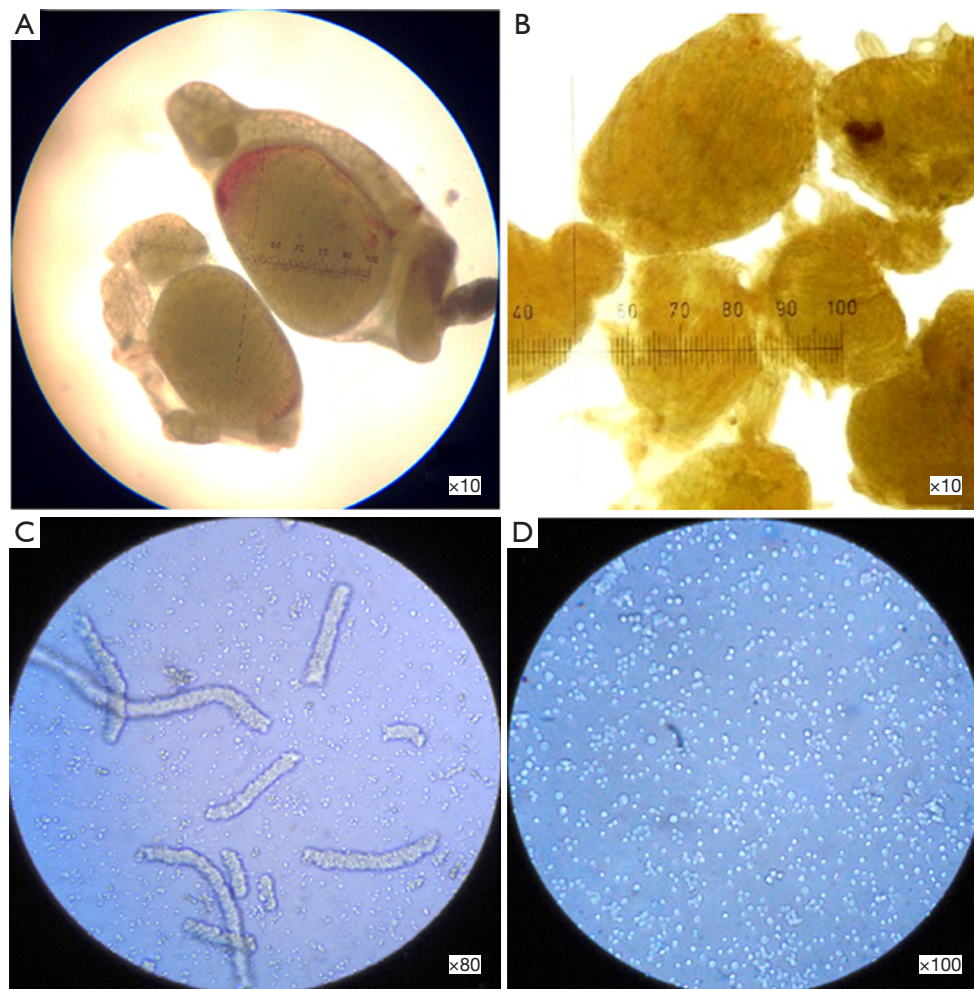
### Study design

This work was an *in vitro* experimental study consisting of testis resection from animals, SSC extraction from the resected testes, freezing-thawing process of the SSCs and then intervention.

### Animals and study groups

A total of 80 6-day-old male BALB/c mice was used for resection of testis. Parents of the animals were kept in standard condition including 12-hour lightness and 12-hour darkness, free available water, free available nutritional concentrate and  $20\pm 2$  °C of temperature. Birthday of the animals was considered as day 0. At day 6, the testes were resected.

SSC extraction was done using magnetic activated cell storing (MACS) technic as reported previously (18). The SSCs were divided into four groups: cryopreservation with selenium intervention (5 and 50 µg/mL), vitrification with selenium intervention (5 and 50 µg/mL), cryopreservation control group (without selenium), and vitrification control group (without selenium). Five µg/mL was regarded as low dose and 50 µg/mL was regarded as high dose.



**Figure 1** SSC extraction procedure. (A) resected testes; (B) removal of Tunica albuginea and epididymis; (C) seminiferous tubes; (D) cellular suspension. SSC, spermatogonial stem cell.

### *Laboratory investigations*

#### **Testis resection**

High-dose ketamine (80 mg/kg) and xylazine (10 mg/kg) were used for anesthesia. Suprapubic incision was performed and after finding urinary bladder, testes were found inside the urinary bladder. Under guide of dissection microscope, the testes were dissected and resected at sterile condition.

#### **Cellular extraction**

The testes were put in a Dulbecco's modification of Eagle

medium (DMEM) containing penicillin/streptomycin 10%. Tunica albuginea of the testes were removed under dissection microscope. Seminiferous tubes were transferred to a cone tube containing DMEM, 2 mg/mL collagenase and 200–500  $\mu\text{g}/\text{mL}$  DNaseI. The tubes were shaken for 15 minutes at 37 °C. The tubes were washed with DMEM for 2 times. An amount of 1 mL trypsin/EDTA was added to the pallet resulted from previous step; then the mixture was pipetted and shaken in order to separate out the cells (200–500  $\mu\text{g}/\text{mL}$  DNaseI was also added). The cellular suspension was filtered with 60-micron nylon mesh (*Figure 1*).

**Table 1** The primers used for real-time PCR

Gene	Primer sequences (5'-3')	Product length (bp)
<i>GAPDH</i>	F: CAATGTGTCCGTCGTGGATCT	208
	R: GTCCTCAGTGTAGCCCAAGATG	
<i>P53</i>	F: GTTTCCTCTTGGGCTTAGGG	255
	R: CTTCTGTACGGCGGTCTCTC	
<i>Caspase3</i>	F: CAGCACCTGGTTACTATTCTC	125
	R: GTTAACGCGAGTGAGAATGTG	
<i>Bax</i>	F: CGAGCTGATCAGAACCATCA	277
	R: GAAAAATGCCTTTCCCTTC	
<i>Fas</i>	F: GAGAATTGCTGAAGACATGACAATCC	314
	R: GTAGTTTTCACTCCAGACATTGTCC	
<i>Bcl2</i>	F: TAAGCTGTACAGAGGGGCT	344
	R: TGAAGAGTTCCCTCCACCACC	

**SSC extraction**

MACS technic was used for extraction of SSCs. SSCs were extracted using antibody-linked microbeads according to guideline of the manufacture (Miltenyi Biotec, Germany). The complete procedure was described previously (19).

**Cell viability**

Trypan blue staining was used to count live cells. Neobar slide was used to count stain negative cells under light microscope. Trypan blue positive cells were considered as dead cells.

**Freezing process**

For cryopreservation, cell containing cryovials were maintained for 20 minutes at 4 °C, 1 hour at -20 °C and 1 day at -70 °C. Then they were stored in liquid azote tank (-196 °C). For vitrification, the cryovials were kept in nitrogen vapor for seconds, and then they were transferred to liquid azote tank.

**Thawing process**

The vials were removed from azote and put on dry ice. Then below half of the vials were put in 37 °C bain-marie for 2–3 minutes. A new culture environment was added to the vials. Then the mixtures were transferred to a falcon containing cell culture environment in 37 °C.

**Gene expression study**

Column-based method was used for extraction of RNA.

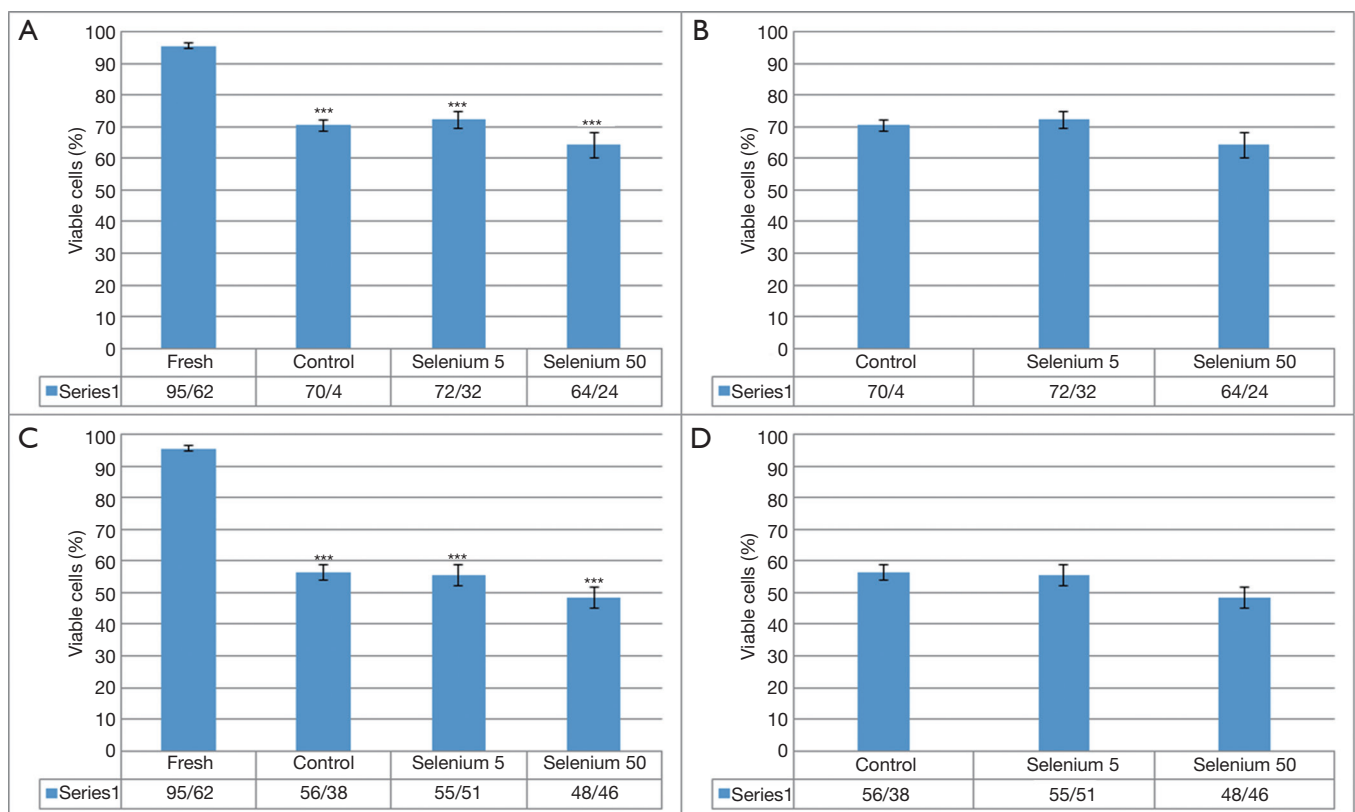
Total RNA purification kit (Jena Bioscience, Germany) was used according to guideline of the manufacture. All equipment were DNase and RNase free. For cDNA synthesis a random hexamer-based kit (Bioneer, South Korea) was used according to guideline of the manufacture. Real-time PCR was used to investigate gene expression. The primers were designed using NCBI and Primer 3 software (Bioinfo, Estonia). *GAPDH* was used as internal control. The primers are shown (*Table 1*). SYBER green master mix was used (Jena Bioscience, Germany). Electrophoresis gel was used to validate the sizes of real-time PCR products. Relative expressions were reported using Rest 2009 software (Technical University, Munich).

**Ethical considerations**

This work was done according to ethical guidelines of working with laboratory animals under supervision of the ethics committee of Lorestan University of Medical Sciences LUMS.REC.1395.162.

**Statistical analysis**

One-way ANOVA was used to investigate significance of differences among the groups of study using SPSS version 21 software (IBM, US). Standard error of means (SEM) was reported as error bar. Gene expression analysis was



**Figure 2** Cellular viability frequency (%). (A) Comparison of fresh cell group and cryopreservation (control and intervention) groups; (B) comparison among the cryopreservation groups including control and intervention groups; (C) comparison of fresh cell group and vitrification (control and intervention) groups; (D) comparison among the vitrification groups including control and intervention groups. \*\*\*, significant at  $P < 0.001$  based on ANOVA. The error bar is SEM. SEM, standard error of means; ANOVA, analysis of variance.

done using Rest 2009 software.  $P=0.05$  was considered as significant level.

## Results

### Viability study

Frequency of viable cells in fresh samples (without freezing-thawing process) was 95.62%. The frequency in cryopreservation control group, cryopreservation group with selenium 5  $\mu\text{g}/\text{mL}$  and cryopreservation group with selenium 50  $\mu\text{g}/\text{mL}$  group were 70.40%, 72.32% and 64.34%, respectively. The reduction of cell viability in these three groups in comparison to the group of fresh samples was significant ( $P < 0.001$ ). Comparison of cell viability these three groups did not show a significant association. Frequency of viable cells in vitrification control group, vitrification group with selenium 5  $\mu\text{g}/\text{mL}$  and vitrification group with selenium 50  $\mu\text{g}/\text{mL}$  group were 65.38%,

55.51% and 48.46%, respectively. The reduction of cell viability in these three groups in comparison to the group of fresh samples was significant ( $P < 0.001$ ). Comparison of cell viability these three groups did not show a significant association. All analyses were done with analysis of variance (ANOVA) (Figure 2).

### Gene expression study

Expression of *Bcl2*, *Bax* and *P53* was not statistically different between cryopreservation control group and the cryopreservation group administered with selenium 5  $\mu\text{g}/\text{mL}$ . However, relative expression of *Fas* and *Caspase3* was significantly lower in the intervention group ( $P < 0.001$ ). Expression of *Fas*, *Caspase3*, *Bax* and *P53* was not statistically different between cryopreservation control group and the cryopreservation group administered with selenium 50  $\mu\text{g}/\text{mL}$ . However, relative expression of *Bcl2* was significantly lower

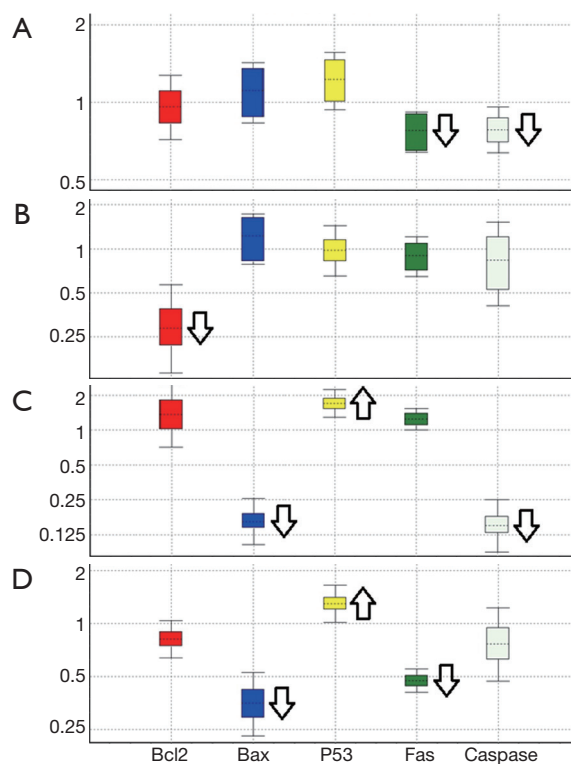
**Table 2** Real-time PCR analysis. Comparison of relative expressions between groups

Gene	Type	Reaction efficiency	Expression	Std. error	95% CI	P value	Result
Cryopreservation—selenium 5 µg/mL vs. control							
<i>GAPDH</i>	REF	0.785	1.000	–	–	–	–
<i>Bcl2</i>	TRG	0.7175	0.955	0.784–1.172	0.728–1.255	0.830	–
<i>Bax</i>	TRG	0.8175	1.090	0.860–1.382	0.837–1.419	0.661	–
<i>P53</i>	TRG	0.6275	1.208	0.975–1.499	0.941–1.551	0.321	–
<i>Fas</i>	TRG	0.725	0.767	0.646–0.909	0.643–0.914	0.000	Down
<i>Caspase3</i>	TRG	0.7475	0.781	0.676–0.906	0.643–0.950	0.000	Down
Cryopreservation—selenium 50 µg/mL vs. control							
<i>GAPDH</i>	REF	0.88	1.000	–	–	–	–
<i>Bcl2</i>	TRG	0.5	0.285	0.189–0.458	0.150–0.552	0.000	Down
<i>Bax</i>	TRG	0.8225	1.162	0.805–1.679	0.785–1.719	0.661	–
<i>P53</i>	TRG	0.8225	0.970	0.762–1.264	0.670–1.414	0.661	–
<i>Fas</i>	TRG	0.865	0.883	0.686–1.140	0.652–1.197	0.661	–
<i>Caspase3</i>	TRG	0.805	0.790	0.480–1.330	0.420–1.494	0.830	–
Vitrification—selenium 5 µg/mL vs. control							
<i>GAPDH</i>	REF	0.71	1.000	–	–	–	–
<i>Bcl2</i>	TRG	0.725	1.335	0.900–2.076	0.743–2.432	0.489	–
<i>Bax</i>	TRG	0.7725	0.162	0.127–0.216	0.106–0.250	0.000	Down
<i>P53</i>	TRG	0.765	1.697	1.434–2.031	1.307–2.210	0.000	Up
<i>Fas</i>	TRG	0.7075	1.241	1.056–1.463	1.010–1.525	0.163	–
<i>Caspase3</i>	TRG	0.7825	0.149	0.114–0.207	0.093–0.245	0.000	Down
Vitrification—selenium 50 µg/mL vs. control							
<i>GAPDH</i>	REF	0.7825	1.000	–	–	–	–
<i>Bcl2</i>	TRG	0.505	0.816	0.702–0.958	0.649–1.030	0.327	–
<i>Bax</i>	TRG	0.8125	0.350	0.269–0.465	0.237–0.519	0.000	Down
<i>P53</i>	TRG	0.7275	1.301	1.134–1.510	1.036–1.640	0.000	Up
<i>Fas</i>	TRG	0.6825	0.474	0.428–0.527	0.411–0.548	0.000	Down
<i>Caspase3</i>	TRG	0.8325	0.764	0.569–1.058	0.487–1.208	0.499	–

The analyses were done in Rest 2009 software. TRG, target gene; REF, reference gene (internal control). Std., standard; CI, confidence interval.

in the intervention group ( $P < 0.001$ ). Expression of *Bcl2* and *Fas* was not statistically different between vitrification control group and the vitrification group administered with selenium 5 µg/mL. However, relative expression of *Bax* and *Caspase3* was significantly lower in the intervention group, and relative expression of *P53* was significantly upper ( $P < 0.001$ ).

Expression of *Bcl2* and *Caspase3* was not statistically different between vitrification control group and the vitrification group administered with selenium 50 µg/mL. However, relative expression of *Bax* and *Fas* was significantly lower in the intervention group, and relative expression of *P53* was significantly upper ( $P < 0.001$ ) (Table 2, Figure 3).



**Figure 3** Relative expression of the genes. (A) Cryopreservation—selenium 5 µg/mL *vs.* control; (B) cryopreservation—selenium 50 µg/mL *vs.* control; (C) vitrification—selenium 5 µg/mL *vs.* control; (D) vitrification—selenium 50 µg/mL *vs.* control.

## Discussion

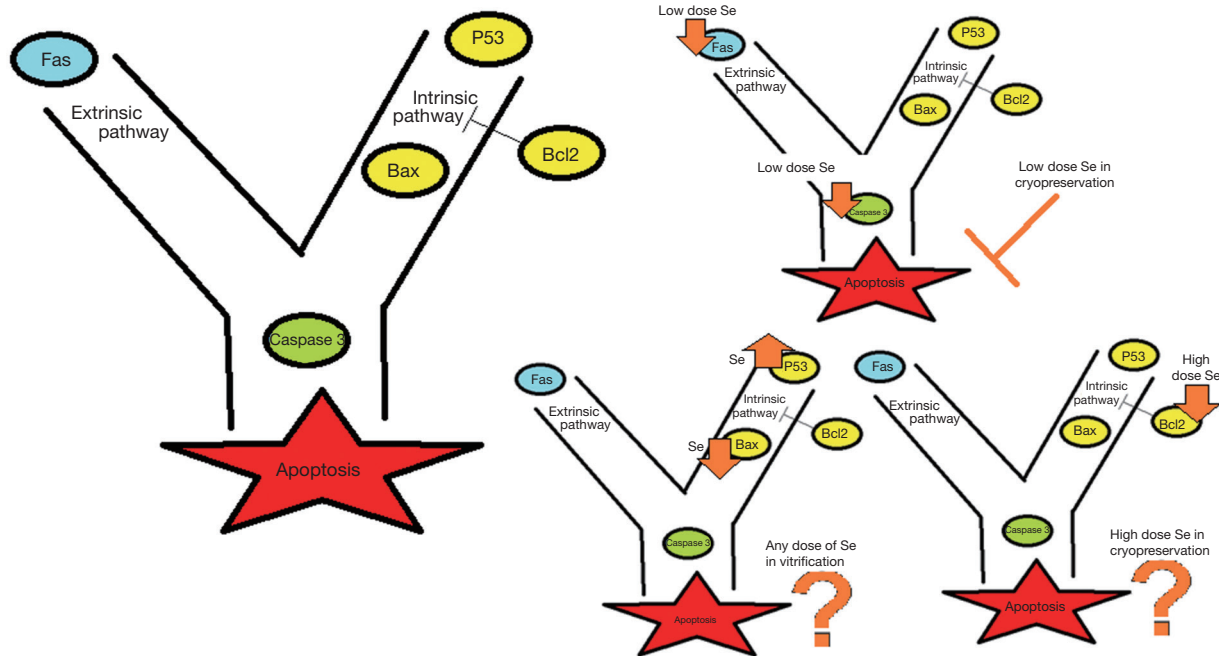
The present study was aimed to investigate the effects of administration of selenium on freezing-thawing damage of SSCs. This damage was investigated using cellular viability and expression of apoptosis related genes. In this regard, trypan blue staining and real-time polymerase chain reaction (real-time PCR) were performed, respectively.

The results of trypan blue staining showed a significant reduction in cell viability for all study groups in comparison to fresh cells. This result showed that this *in vitro* modeling of cellular damage was performed correctly. The results of real-time PCR showed a dose-dependent effect for selenium on expression profile of apoptosis related genes. Briefly, selenium in both doses resulted in up regulation of *P53* and down regulation of *Bax* in vitrification intervention group, as well resulted in down regulation of *Caspase3* in low-dose intervention both in cryopreservation and vitrification intervention groups. Down regulation of *Bcl2* occurred only in high-dose intervention of selenium in cryopreservation

group. Down regulation of *Fas* occurred in low-dose intervention of selenium in cryopreservation group and high-dose intervention of selenium in vitrification group.

Among the apoptosis related proteins, *Bcl2* is inhibitor of apoptosis whereas *Fas*, *Bax*, *P53* and *Caspase3* are the activators. Among them, *Fas* is for extrinsic pathway while *P53* may affect both pathways. Of course, *Caspase3* can be considered as a common pathway (20,21). Our study showed different effects of selenium on these pathways in different doses and different conditions according to real-time PCR study. Low-dose selenium in cryopreservation may inhibit apoptosis via inhibition of extrinsic pathway; however, this inhibition was not significant according trypan blue staining. High-dose selenium inhibits *Bcl2* in cryopreservation; however, the outcome on apoptosis is not clear. In vitrification condition both low and high doses of selenium up regulate *P53* and down regulate *Bax* (in low dose *Caspase3* is down regulated and in high dose *Fas* is down regulated); however, the outcome on apoptosis is not clear, because the effect on *P53* and *Bax* is contradictory. The scheme of these findings is shown (Figure 4).

History of investigation of the effects of selenium on spermatogenesis and male reproduction system backs to the previous century. Saaranen *et al.* (1989) investigated selenium content of semen on mitochondrial sheath of sperms in human and animals. They found no significant correlation in human (22). Presence of selenium in semen give researchers this rationale to use selenium in *in vitro* studies. Younis *et al.* (1998) investigated the effects of insulin transferrin selenium (ITS) combination on cryopreservation of Chimpanzee spermatozoa and they found better sperm motility. In contrast, our study was on SSCs instead of spermatozoa (23). Wu *et al.* (2009) performed a study on cryopreservation of rat SSCs. They used a combination which one of its components was selenium. Investigation of gene study had been suggested (24). Many other studies had investigated the effects of ITS on cryopreservation; however, there was no study on using selenium alone on freezing-thawing damage based on gene expression study. Rezaeian *et al.* (2016) investigated the effects of selenium on human sperm parameters in frozen-thawed samples. They showed that low-dose selenium administration could increase sperm motility (25). Pourmasumi *et al.* (2018) conducted a clinical trial. They found that combined supplementation of vitamin E and selenium sperm parameters (26). Ghafarizadeh *et al.* (2018) investigated effects selenium supplementation on sperm quality of asthenoteratozoospermic men. They found that



**Figure 4** Effects of different doses of selenium (Se) on apoptosis pathways in cryopreservation and vitrification conditions according to real-time PCR results. For P53, its role in the intrinsic pathway was regarded in this graph.

*in vitro* selenium supplementation might protect sperms from ROS (27). Recently (2019), effects of selenium nanoparticles have been investigated in bull sperm cryopreservation and they found positive results (28).

Many *in vivo* studies have shown effects of selenium on animal oxidative stress models. Kaur and Bansal (2015) investigated effects of selenium on mice scrotal hyperthermia induced oxidative stress and they found protective effects via antioxidant and anti-apoptotic effects (16). Kara *et al.* (2016) showed protective effects of selenium on testicular ischemia-reperfusion injury of rat via antioxidant and anti-apoptotic effects (29). Kaur *et al.* in another study (2018) showed that selenium had antioxidant and anti-apoptotic effects in bisphenol A induced oxidative stress model of mice (30).

Briefly, selenium is an important element in semen and male fertility. *In vivo* studies showed protective effects of selenium on different animal models of testicular injury. *In vitro* studies showed that selenium and selenium containing compounds had protective effects on the sperms undergone freezing-thawing damage. In contrast, in the present study, selenium could not increase cellular viability of the SSCs undergone freezing-thawing damage. However, selenium could influence apoptosis related genes expression profile.

Since SSC preservation process in children with cancer is an *in vitro* process, the evidence of using the results in clinics should be based on such studies.

### Limitations

Lack of significant association in cellular viability analysis may be due to low power of the design of our study. Lack of cell culture timeline can be pointed out; because gene expression merely shows behavioral tendencies of cells, and it takes time to show clinical manifestation.

### Conclusions

Administration of selenium could not increase cellular viability of SSCs after freezing-thawing damage of cryopreservation and vitrification procedures. According to gene expression study, selenium had dose-dependent effect on apoptosis related genes profile. The only evident effect was the effect of low-dose selenium in cryopreservation on inhibition of apoptosis via extrinsic pathway. Therefore, *in vitro* administration of selenium to SSCs of the children undergone cancer treatment may not be clinically beneficial based on the current evidence.



## Acknowledgments

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## Footnote

*Conflicts of Interest:* The authors have no conflicts of interest to declare.

*Ethical Statement:* The authors are accountable 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. This work was done according to ethical guidelines of working with laboratory animals under supervision of the ethics committee of Lorestan University of Medical Sciences (IR.LUMS.REC.1395.162).

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