Original Research

Increasing of post-freezing quality of Spermatogonial Stem Cells after pretreatment by vitamin E

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Received: January 2019; Accepted: January 2019; Published online: March 2019

Abstract: **Introduction:** Mouse spermatogonial stem cells (SSCs) can be cryopreserved for long periods while preserving their spermatogenic ability. Although cryopreservation has been found to increase reactive oxygen species (ROS) formation that damages cellular structures. In the present study, we added vitamin E to the basic freezing medium in order to evaluate its effect on the efficiency of spermatogonial stem cells.

Methods: SSCs isolated from testes of 6 days old male mice by enzymatic digestion. Vitamin E 100, 200, 400 µg/mL was added to the basic freezing medium. The cell viability was evaluated by MTT assay. After thawing, SSCs were cultured for 1 month and the expression pattern of specific genes of SSCs measured by real-time PCR technique.

Results: The survival rate of the freeze cells in the presence of vitamin E was significantly higher than the control group ($p<0.05$). The number of colonies and their diameter measured after one month were significantly higher in the vitamin E groups than in the control group ($p\leq0.05$).

Conclusion: Adding vitamin E to the basic freezing medium thus can be helpful in increasing the quality and viability of SSCs after cryopreservation.

Keyword: Spermatogonial stem cell; Cryopreservation; Culture; vitamin E

Cite this article as: Aliakbari F, Heidari MH, Hossini MA, Hossini J. Increasing of post-freezing quality of Spermatogonial Stem Cells after pretreatment by vitamin E. Men's Health Journal 2019; 3 (1); e1.

1. Introduction

Spermatogenesis is one of the most proliferative and active processes of mammalian males (1). Spermatogonial stem cells (SSCs) play a fundamental role through spermatogenesis and are unique among adult stem cells in animals in the term of possessing the ability to transmit genetic information to the next generation (2). SSCs are an attractive target for a wide spectrum of researches, genetic manipulation, cryopreservation and transplantation studies due to their specific characterizations including, maintenance of euploid karyotypes, normal methylation patterns, and germline competence during long-term culture (3). Meanwhile, the ability of SSCs to self-renew makes them a promising alternative for genetic preservation of rare and endangered animals (4).

Male fertility preservation should be suggested before starting chemotherapy and radiotherapy treatment in cancer patient because these procedures can permanently damage fertility (5). Because SSCs also appear early during the postnatal period then they can be collected not only from adults but also from reproductively immature animals (6). Some new strategies for male infertility preservation have been suggested such as testicular cell suspension or testicular tissue freezing (5, 7). SSCs can be cryopreserved using simple cryopreservation techniques as first demonstrated in the mouse (8). Combination of culture approach with cryopreservation has been found to be a suitable approach for long-term preservation of SSCs, and by increasing the number of SSCs; transplantation technique can then be used to restore spermatogenesis in the infertile male. Methods

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used for cryopreservation of SSCs are similar to those used for somatic cells; however, there are a few studies on the evaluation of cryopreservation effects on SSCs. It is well established that cryopreservation procedure increases reactive oxygen species (ROS) formation, generation of free radicals causing oxidative damage and finally cell death (9, 10). ROS are highly reactive molecules derived from oxygen metabolism and lead to great destruction in cell membranes and DNA by causing membrane lipid peroxidation and decreased membrane fluidity (11). Antioxidants are important elements in keeping the redox status inside tissues and cells and several studies have reported that vitamin E plays a critical role in decreasing ethanol-induced oxidative stress by acting as a proton donor to scavenging ROS (7, 12). Vitamin E is considered to be one of the most effective antioxidants, with remarkable scavenging action against peroxyl radicals that protects molecules such as lipids, proteins, and nucleic acids against oxidation (13).

SSCs similar to other stem cells are generally in scarce population, then the success rate of transplants depends on the enrichment and concentration of transplanted SSCs in vitro (14). The culture of SSCs in vitro enhances SSCs numbers and probability of successful transplantation as well as their detailed characterization, recognition of involved molecules in SSCs self-renewal and differentiation capacity (15, 16). The aim of this study was to compare effects of different doses of vitamin E on the cryopreservation of SSCs obtained from mouse testis.

2. Method

2.1. Ethical statements

All experiments were approved by the ethics committee of Beheshti University of Medical Sciences and performed in accordance with the university guidelines.

2.2. Animals

Six-days old NMRI mice were maintained under 12 hrs light/12 hr dark cycle laboratory conditions with free access to food and water.

2.3. Testis Cell Collection

In order to isolate SSCs from testis tissue, two-step enzymatic digestions were used (6 mice for each group). Testes were collected and after removing additional tissues, were washed in phosphate buffered saline (PBS; Sigma, Germany). After removing of tunica albuginea, testes were minced into small pieces and transferred to the digestion solution containing collagenase type IV (1 mg/mL, Sigma, Germany), DNase (10 µg/ml, Sigma, Germany) and hyaluronidase (0.5 mg/mL, Sigma, Germany) in minimum essential medium alpha (αMEM; Sigma, Germany) for 20 min, with slow shaking at 37°C in water bath until the tubules were disassociated. The cells were then centrifuged at 1500 g for 5 min after which they were washed twice with PBS. Second step digestion was performed for testis cells suspension with the same enzymes used above (15 min) and the digested cells were then washed with PBS.

2.4. Cryopreservation

Following cell isolation, freezing media were slowly added to the cell suspension. This experiment had four groups :1) Control group: the basic freezing medium consisted of dimethyl sulfoxide (DMSO;1.4M, Sigma, Germany), 10% fetal bovine serum (FBS; Sigma, Germany), and MEM-α (Sigma, Germany) (17) and vitamin E group: 100, 200, 400 µg/mL vitamin E (Sigma, Germany) which was added to the basic freezing medium. Cryovials were placed into CryoBox (Nalgene, Z359017, Sigma) and stored in −80 °C freezer for at least 1 day. After overnight storage, cryovials containing frozen cells were immersed into liquid nitrogen for minimum one week.

2.5. Thawing procedure

After one week, cryovials were removed from liquid nitrogen and were kept at room temperature for 30 secs and then in a water bath at 37°C for 2 min. The cryovials contents were transferred to a tube with a pre-warm medium (MEM +⁄10FBS). The cells were washed and centrifuged at 1200g for 5 min (17). The supernatant was removed and the cell pellet was used for several assessments including viability, gene expression by real-time PCR and cell culture.

2.6. Cell viability

Cell viability was evaluated by methylthiazoltetrazolium (MTT; Sigma, Germany) assay in control and treated group with vitamin E. 400µl MEM and 40µl MTT were added to each 400 cells-contained well for each group and incubated for 4 h at 37°C followed by replacing medium by 400µl DMSO. The cells were maintained at room temperature for 30 min and the optical density (OD) at 540 nm was measured using a microplate reader.

2.7. Intracellular ROS measurement

CM-H2DCFDA (General Oxidative Stress Indicator) is able to detect intracellular H_2O_2 and was applied for intracellular ROS measurement in control and treated group with vitamin E. DCFDA (10 µl, Sigma, Germany) was added to cells and incubated at 37 °C for 25 min. The cells were twice washed with PBS following centrifuging at 2500g for 5 min. Green fluorescence was measured by flow cytometry between 500 and 530 nm (18).

2.8. SSCs culture

SSCs were cultured for one month after cryopreservation in all groups (density of 2 × 10^5 cells/cm^2). Basic culture medium contained MEMα supplemented by 10% FBS, 1 x nonessential amino acids (Invitrogen, USA), 0.1 mM 2-mercaptoethanol (Sigma, Germany), 10^3U/ml human recombinant leukemia inhibitory factor (LIF; B&D, USA), 100U/ml penicillin and 100 µg/ml streptomycin (both from Sigma, Germany). 10µg/ml glial cell line-derived neurotrophic factor (GDNF; R&D, USA) was added to each well. The culture medium was replaced every 3 days by fresh medium. All cultures were maintained at 32˚C in an atmosphere humidified with 5% CO_2 and cells were sub cultured every 7 to 10 days using trypsin-EDTA (Gibco).

2.9. Colony assay

The diameter and number of colonies were assessed at the end of the first, second and fourth week (7, 14 and 30 days after seeding). The colony formation initiated earlier in 4 days in treated groups but this was observed in 7 days in control. We count all colonies in each 30 mm dishes in three independent experiments and colonies diameters were measured using an inverted microscope equipped by ocular grid and images were processed by imageJ software (19).

2.10. Real-time PCR

for the evaluation of optimal conditions for SSCs cryopreservation, immediately after cryopreservation and after one month of culture, the transcripts levels of apoptosis regulator BAX (*Bax*, pro-apoptotic) gene, B-cell lymphoma 2 (*Bcl2*, anti-apoptotic) gene, tyrosine-protein kinase Kit (*c-kit*, differentiated gene), promyelocytic leukemia zinc finger protein (*Plzf*, undifferentiated gene) and DNA-binding protein inhibitor ID-4 (*ID4*, undifferentiated gene) were evaluated by Real-time PCR. The primer sequences have been shown in (Table1). Total RNA was extracted by RNase Kit (Ready Mini Kit, Qiagen, USA), according to the manufacturers catalog. cDNA synthesis was performed by reverse transcription kit (Transcript First Strand cDNA Synt, Roche, USA) with 1µg of total RNA, according to the manufacturer's catalog. Real-time PCR was performed with forty reaction amplification cycles and Applied Bioscience 7500 fast with SYBR Green detection was used for the analysis. All samples were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (internal control) using the comparative CT method (ΔΔCT).

2.11. Statistical analysis

The statistical significance between the mean values was determined by one-way analysis of variance (ANOVA) followed by a Tukey *posthoc* test and data were expressed by as mean ± standard deviation (SD). Level of p≤0.05 was considered as statistically significant and three independent experiments were performed in each group.

3. Result

3.1. SSCs viability

MTT assay results showed that after one week of cryopreservation, the survival rate of cryopreserved cells in the control group (65.43%) was significantly lower than of vitamin E groups with 100, 200 and 400 µg/ml doses (74.54%, 89.65%, 79.13%). Furthermore, cell viability was significantly increased in the 200 µg/ml vitamin E (89.65%) is compared with other vitamin E doses (Figure 1).

3.2. Intracellular ROS measurement

Results showed that after one week of cryopreservation, ROS production in the control group (68.99%) is higher than of vitamin E groups with 100, 200 and 400 doses (45.54%, 29.75%, 38.13%) and also ROS production in vitamin E group with 200 µg/ml doses (29.75%) is less than other vitamin E doses (Figure 2).

3.3. SSCs culture

Obtained SSCs from enzymatic step were immediately cryopreserved and after thawing. Cells were cultured for one month. At the first day following thawing, SSCs

Table 1: Primer sequence.

Gene name	Sequence	temp (0C)
Bcl2	For: 5'-CTGGATCCAAGACCAGGGTG-3' Rev: 5'-CCTTTCCCCTTCCCCCATTC-3'	**58.18**
Bax	For:5'GGGGTCATGTGTGTGGAG-3' Rev: 5'-TCACTTGTGGCCCAGGTA--3'	**57.16**
ID4	For: 5'- CGTTGGGGGTCAGCTAGAAAG -3' Rev: 5'- CACCATGATGACCACATCGC-3'	**61.52**
Plzf	For: 5'- TCCCGCCCAACAAGAAAGTC -3' Rev: 5'- TCAGCAAAGCAGGGTGAGTC-3'	**58.16**
c-kit	For: 5'- AACAACAAAGAGCAAATCCAGG -3' Rev: 5'- GGAAGTTGCGTCGGGTCTAT -3'	**59.60**
GAPDH	For: 5'-AGCAAGGACACTGAGCAAGAG-3' Rev: 5'- TCGTTCCTCTGATCGTTTCC -3'	**61.53**

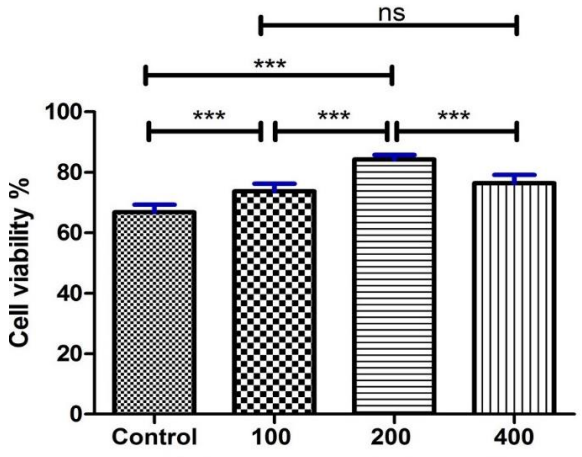

Figure 1: MTT assay analysis of viability in control and treated groups. Results indicated that the survival rate of cryopreserved cells in the control group was significantly lower than in treated groups. Data s show means ± SD; (***) p ≤ 0. 001.

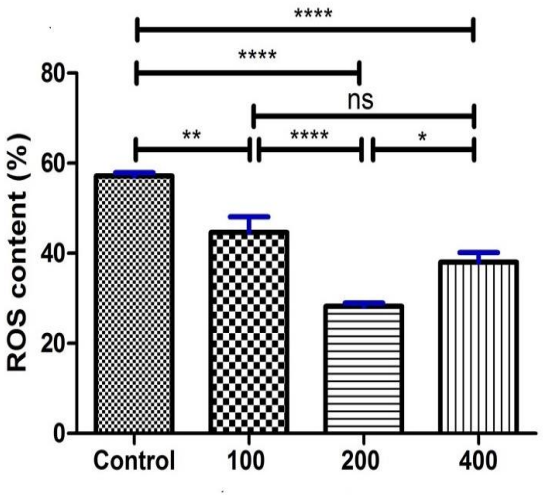

Figure 2: ROS production in control and vitamin E groups. Data show means ± SD; (*) ≤0.05, (**) ≤ 0.01, (***) ≤ 0.001, (****) ≤ 0.0001.

were a round shape with average size gradually started to attach to the dish bottom, however, colonies were apparent after 4-6 days. Formation of cell clusters in vitamin E groups occurred on day 4, whereas, this happened on 6 days in the control group.

3.4. Colony assay

Results of diameter and number assay of colonies showed that following 4 weeks of culture, SSCs formed round colonies with certain border. Diameter and number of colonies in 200 µg/ml vitamin E were significantly more than control, 100 and 400 µg/ml vitamin E (Figure 3).

3.5. Gene expression

Gene expression analysis of SSCs after cryopreservation indicated that the transcript levels of Bax and Bcl2 in the 200 µm/ml vitamin E group was significantly lower and more than other groups, respectively. Expression of ID4 and Plzf genes in 200 µm/ml vitamin E group was higher and differentiation marker of c-kit had a decreased level of expression in 200 µm/ml vitamin E is compared with the other groups (Figure 4a).

After one month of culture, results showed that the level of Bax expression in the 200 µm/ml vitamin E group was significantly lower, however, the level of Bcl2 expression was significantly higher than the other groups. Undifferentiation related genes, ID4 and Plzf, had an increased level of expression in 200 µm/ml vitamin E group, whereas the c-kit gene had a decreased gene expression (Figure 4b).

4. Discussion

In this study, we evaluated a modified cryopreservation protocol for the freezing of mouse SSCs using vitamin E. Our results indicated that cryopreservation of SSCs in the presence of vitamin E can increase cell viability, reduce ROS production and maintaining SSCs undifferentiated state after culture.

Cryopreservation with vitamin E probably indicates the lethal effect of cooling on SSCs is lower. Our results about cell viability by MTT test showed that cryopreservation of SSCs with 200 µm/ml vitamin E have the higher response in compared with other groups than the control group. Avarbock et al. 1996 show that cryopreservation of SSCs is feasible but there are many problems with this method (8). On the other hand, Kanatsu-Shinohara et al demonstrated after cryopreservation, the number of viable cells can decrease because of toxic products of metabolism accumulated during freezing presses (20). We with adding vitamin E to the basic freezing medium showed that cells viability can increase. Studies reported that adding cryoprotectant such as glycerol or DMSO to the freezing medium in order to evaluate the survival of SSCs and they showed that SSCs can be cryopreserved successfully (21). Previous studies indicated that antioxidants can be used in order to reduce the toxic effects ROS molecule during cryopreservation (22). In the current study, we used vitamin E freezing medium to preventing from ROS production. We showed that vitamin E reduce ROS production in treatment group higher than the control group. Vitamin E is a lipid-soluble antioxidant can maintain membrane components from ROS damaging effects (23).

Before and after culture, we analyzed Bax and Bcl2 gene expression and our finding demonstrated that by using vitamin E the apoptotic rate of cells was decreased in

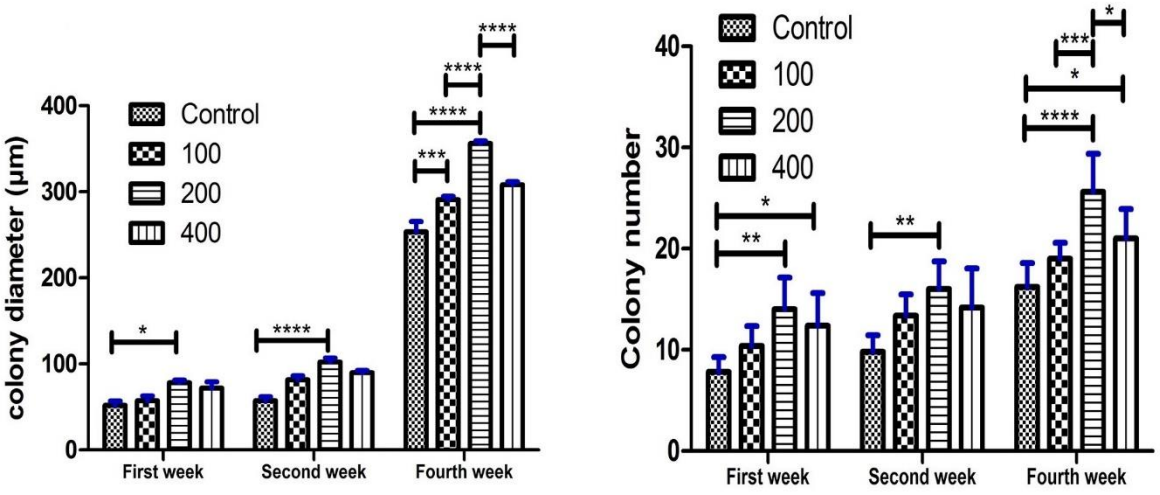

Figure 3: Comparison of colony diameters and number between control and Vitamin E groups. Data show means – SD; (*) ≤ 0.05, (**) ≤ 0.01, (***) ≤ 0.001, (****) ≤ 0.0001.

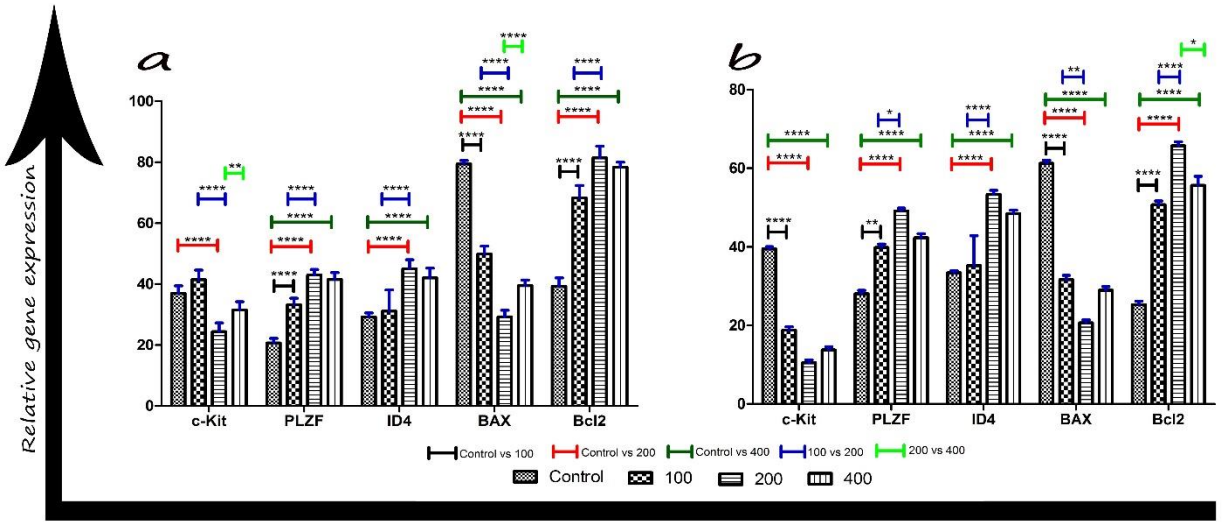

Figure 4: Expression pattern of c-kit, Plzf, ID4, Bax, and Bcl2 genes before (a) and after culture (b), Data show means ± SD;(*) ≤ 0.05, (**) ≤ 0.01, (****) ≤0.0001.

treatment group more than control group. Actually, vitamin E can break the covalent links that ROS forms between fatty acid chains in lipid membranes and reduce apoptosis rate in SSCs (23).

Due to the limited number of SSCs, and the importance of their abundant use, especially in the treatment of infertility in men who have undergone anticancer treatment before puberty, has been widely researched by SSCs in the culture medium (24). In this study, we also cultured them for one month after freezing of SSCs, and cells were evaluated for apoptosis. Meanwhile, we evaluated SSCs markers expressions such as cKit, Plzf and ID4 in the treatment and control group. SSCs colonies in treated groups with vitamin E increased Plzf and ID4 expression and decreased c-kit expression than the control group. The number of colonies and their diameters in cryopreserved groups with vitamin E higher than the control group. This show with adding vitamin E to basic freezing medium probably can cause increases of colony formation and maintain activity gene undifferentiated Plzf and ID4 in SSCs. Past studies indicated that the number and diameter of the colonies increased after 4 weeks of culture and our results agreed with previous studies (25). On the other hand, SSCs are partly resistant to freezing media compared with other spermatogenic cells (25). In the recent study after the freezing process apoptosis gene (Bax, Bcl2) were studied. Bcl-2 family proteins with Bax proteins play a major role in the reg-

ulation of apoptosis. The Bcl-2 gene promotes the cellular death of some cells, such as lymphocytes, and is essential for the survival of cells (26). The Bax gene plays a crucial role in the pathway of cell death. In this study, the expression of BAX and Bcl2 in vitamin E decreased and increased, respectively. Our study clearly provides evidence that vitamin E was most effective in media solution maintaining SSCs.

5. Conclusion

Adding vitamin E to the basic freezing medium thus can be helpful in increasing the quality and viability of SSCs after cryopreservation.

6. Acknowledgment

The authors are thankful to Dear colleagues of the Infertility & Reproductive Health Research Center and Mahdieh Hospital for providing financial assistance and research facilities to conduct this project.

7. Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

8. Funding support

This study was supported by a research grant from Men's Health & Reproductive Health Research Center (MHRHRC), Shahid Beheshti University of Medical Sciences.

9. Author's contributions

All the authors have the same contribution.

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