TÜBİTAK

Turkish Journal of Veterinary and Animal Sciences

http://journals.tubitak.gov.tr/veterinary/

Research Article

Turk J Vet Anim Sci
(2017) 41: 648-655
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doi:10.3906/vet-1701-38

The effect of combining vitamin E and C on the viability improvement of transfected ovine spermatogonial stem cells after cryopreservation and thawing

Hosein SHABANI[1], Mohammad ZANDI[1,*], Hamideh OFOGHI[2], Mohammad Reza SANJABI[1], Khosro HOSEINI PAJOOH[2]

[1]Department of Agriculture, Iranian Research Organization for Science and Technology (IROST), Tehran, Iran
[2]Department of Biotechnology, Iranian Research Organization for Science and Technology (IROST), Tehran, Iran

Received: 13.01.2017 • **Accepted/Published Online:** 20.07.2017 • **Final Version:** 13.11.2017

Abstract: The present study aimed to identify whether vitamin E and C and their combination in basal freezing medium improved the viability of postthaw transfected spermatogonial stem cells (SSCs). For this purpose, SSCs were harvested by enzymatic digestion twice. After enrichment and culture on Sertoli cell feeder layer, SSCs were characterized by using alkaline phosphatase staining and expression of *oct-4* and *c-kit* genes as specific stem cell markers. As to the transfection of SSCs, different concentrations of DNA (0.2, 0.4, and 0.8 µg) and turbofect (0.4 and 0.8 µL) in 100 µL of medium were studied. The results showed that cryopreservation of SSCs in the presence of 25 µg/mL vitamin E and 10 µg/mL vitamin C could increase cell viability and expression of antiapoptotic genes. The best combination of DNA and turbofect for transfection of SSCs in 100 µL of medium was 0.8 µg and 0.4 µL, respectively. However, SSCs indicated lower transfection efficiency compared with Sertoli cells (~30% vs. 70%, respectively). Cryopreservation with the addition of vitamin E and C in the basal freezing medium could increase cell viability of transfected SSCs as well. The findings of this study also suggest the need for further research regarding improvement of the transfection efficiency of SSCs.

Key words: Spermatogonial stem cells, cryopreservation, transfection, vitamin E, vitamin C, sheep

1. Introduction

The male germline has made the majority of genetic gain in livestock populations (1). Spermatogonial stem cells (SSCs) as postnatal male germline stem cells can be transplanted into the recipient testis and give rise to a vast number of spermatozoa producing offspring (2). Thus, in farm animals, combining male germ cell transplantation with a large-scale culture of SSCs would provide a renewable source of stem cells for transplantation (3). This system has great potential since it provides proper alternatives to artificial insemination (AI) for the use of elite sires in an extensive production system in which AI is more impractical (4). Moreover, SSCs can be utilized in gene transfer to the next generation and this is proved to be more effective and time-saving than other approaches such as the use of embryonic stem cell or nuclear transfer technologies (5).

Recently, SSC transplantation has been applied to livestock species, initially in pigs, goats, and cattle (2,3). However, no male germ stem cell line has yet been established for livestock, which limited stem cell studies on farm animals (2,6).

In vitro culture and cryopreservation of SSCs are integral parts of the application of this technology (7). The applied methods for cryopreservation of SSCs are similar to those of somatic cells (8). However, recent findings on SSC cryopreservation appear not to be satisfactory. Freezing can increase the formation of reactive oxygen species (ROS), resulting in oxidative stress on the cells. Moreover, SSCs sustain large volume changes due to the various osmotic pressures during freezing (9).

To prevent oxidative stress damage to cellular structures, it is necessary to have a complex network of antioxidants and enzymes (8). Two different kinds of antioxidants are known: enzymatic (such as catalase and superoxide dismutase) type and nonenzymatic (such as vitamin E and C) type (9).

The effects of vitamin E have been taken into account by its antioxidant functions, which involve the scavenging of ROS as well as reactive nitrogen species (8). Vitamin E is used for the prevention of the peroxidation of unsaturated lipids in cell membrane and protection of intracellular phospholipids. During cryopreservation, vitamin E positively affects the membrane integrity of cryopreserved cells (9). However, contradictory effects on different cell

* Correspondence: mz1075@yahoo.com

types may result through supplementation with different concentrations of vitamin C. By adding vitamin C at specific concentrations in the culture medium, it can be utilized as a growth promoter since it can result in increasing cell proliferation and DNA synthesis. However, high concentrations of vitamin C are cytotoxic, which appear to result in inhibition of proliferation and apoptosis (6).

Despite remarkable attempts regarding the development of a culture system in order to maintain and proliferate SSCs in vitro, there seems to be a need to establish an effective SSC culture system for sheep. It is also noteworthy that little to no research has been carried out concerning the in vitro culture conditions for longtime maintenance of ovine SSCs (6).

Hence, the present study initially investigated the effect of various concentrations of vitamin E and C alone or in combination on cryopreservation of ovine SSCs. Secondly, it was aimed to see whether vitamin E and C alone or in combination in culture of ovine SSCs could improve the viability of transfected SSCs after cryopreservation and thawing.

2. Materials and methods

2.1. Chemicals

Unless otherwise mentioned, all chemicals used in this work were purchased from Sigma (St. Louis, MO, USA) and media were obtained from Gibco (Life Technologies, Rockville, MD, USA).

2.2. Isolation and culture of SSCs

All animal procedures and the experimental protocol of this study were approved by the animal care and use committee of the Iranian Research Organization for Science and Technology (IROST). Lambs' testes (3 to 5 months old) were collected from a local slaughterhouse and brought to the laboratory within 2 h of slaughter. SSCs were isolated by a two time enzymatic digestion process as described by Izadyar et al. (10) with some modifications. For the first enzymatic digestion, minced seminiferous tissue was suspended in Dulbecco's modified Eagle medium (DMEM) containing 1 mg/mL trypsin (Invitrogen), 1 mg/mL hyaluronidase type II, 1 mg/mL collagenase, and 5 µg/mL DNase and was incubated at 37 °C in a shaker incubator (200 cycles/min) for 45 min. The dispersed tissue was collected and subjected to centrifugation at 1000 rpm for 2 min. The supernatant was collected and the pellet was washed with DMEM. For the second enzymatic digestion, the pellet was suspended in DMEM containing 1 mg/mL hyaluronidase type II, 1 mg/mL collagenase, and 5 µg/mL DNase and incubated in a shaker incubator (200 cycles/min) for 30 min. The pellet was then centrifuged at 1000 rpm for 2 min.

2.3. Enrichment of SSCs

For enriching SSCs, the supernatant was filtered through an 80-µm and then a 60-µm nylon net filter. The filtered cells were then transferred to lectin–bovine serum albumin (BSA)-coated 35-mm petri dishes. The lectin–BSA-coated dishes were prepared by dissolving lectin (5 µg/mL) from *Datura stramonium* agglutinin in Dulbecco's phosphate-buffered saline (DPBS). The dishes were kept for 2 h at room temperature, after which they were washed with BSA (0.6% BSA in DPBS). The dishes were then kept at room temperature for another 2 h for coating BSA. The cells seeded on the lectin-coated dishes were incubated for 5–6 h at 37 °C in a CO_2 incubator with 5% CO_2 in air to enable most of the contaminating cells to get attached to the lectin–BSA. After that, the remaining medium, which was expected to contain SSCs, was collected and transferred to a 15-mL tube. It was then centrifuged for 5 min at 1000 rpm, following which the supernatant was discarded and the pellet was resuspended in DMEM. These cells were then subjected to Percoll density gradient cell separation for further purification based on van Pelt et al.'s (11) method.

2.4. Preparation of feeder layers

The cells left over in the lectin-coated dishes were revitalized with fresh DMEM supplemented with 10% FBS and were incubated in a CO_2 incubator with 5% CO_2 in air at 37 °C for 2–3 days to enable these cells, which were expected to be primarily Sertoli cells, to grow until a confluent monolayer was formed. For propagation, the cells were subcultured in a 25-cm culture flask after being disaggregated with 0.25% trypsin–EDTA. For the preparation of a feeder layer, Sertoli cells were inactivated by treatment with 10 µg/mL mitomycin-C for 3 h and were subsequently washed 5 times with DPBS and finally with DMEM supplemented with 10% FBS.

2.5. Culture of SSCs

The collected SSCs were seeded on the Sertoli cells feeder layer in 35-mm dishes and were incubated in a CO_2 incubator with 5% CO_2 in air at 37 °C. The culture medium was replaced every third day with DMEM medium supplemented with 10% FBS. SSC colonies were observed after 10 days.

2.6. Characterization of SSCs

Alkaline phosphatase staining and expression of *oct-4* and *c-kit* genes were used for characterization of SSCs. For alkaline phosphatase staining, SSC colonies were washed twice with DPBS and then stained using an alkaline phosphatase kit as per the manufacturer's protocol.

2.7. RNA isolation, reverse transcription. and PCR

Total RNA was isolated with TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA) and was subsequently treated with DNAse (Ambion Inc., Houston, TX, USA) to avoid

DNA contamination. Reverse transcription was done with MMLV enzyme and oligo dT primers (SinaClon Co. Tehran, Iran). PCR reactions were set up in a final volume of 25 µL having 10X PCR buffer (SinaClon Co. Tehran, Iran), 10 mM dNTPs (SinaClon Co. Tehran, Iran), 1.0 U of GoTaq DNA polymerase (SinaClon Co. Tehran, Iran), and 10 µM each of forward and reverse primers. The PCR cycling conditions for the various genes under study were 94 °C for 3 min, followed by a cycling program of 94 °C for 30 s, primer-specific annealing temperature (as mentioned in Table) for 30 s, and 72 °C for 30 s and 32 cycles. The reactions were ended with a final extension at 72 °C for 10 min.

2.8. Transfection of Sertoli and SSC cells

The enhanced green fluorescent protein (EGFP) plasmid construction (pEGFPN1, 5.4 kb) used in this experiment contained the CMV promoter. Plasmids were extracted using a Plasmid Mega Kit (Qiagen Inc., Valencia, CA, USA) and the StuI restriction enzyme (Takara Bio., Shiga, Japan) was used to prepare linearized plasmid following the manufacturer's instructions, and digestion efficiency was checked by 1% agarose gel electrophoresis. One day before transfection, 0.5×10^5 Sertoli cells were plated in 100 µL of growth medium (DMEM and 10% FBS) so that the cells were at 75% confluency at the time of transfection. Different concentrations of DNA (0.2, 0.4, and 0.8 µg) and turbofect (Thermo Fisher Scientific, Rockford, IL, USA) (0.4 and 0.8 µL) were diluted in 50 µL of transfection medium, separately, and incubated for 5 min at room temperature. Then the diluted DNA was added to diluted turbofect (total volume = 100 µL) and incubated for 20 min at room temperature. Next, 100 µL of complexes was added to each well containing cells and mixed gently by rocking the plate back and forth. The cells were then incubated at 37 °C in a CO_2 incubator for 24 h prior to testing for transgene expression by fluorescence microscopy (Nikon Eclipse TE 300, Tokyo, Japan).

2.9. Cryopreservation

Freezing medium (10% DMSO + 10% FBS + 80% DMEM) was prepared and sterilized through a 0.22-µm syringe filter. Cell pellets were resuspended in 1 mL of medium according to experimental design and transferred to the cryovials (Corning Glass Works, Corning, New York, NY, USA). The cryovials were subjected to slow freezing in a freezing container (Mr. Frosty, Nalgene Nunc International, Rochester, NY, USA) at –80 °C for 24 h. When the cryovials attained –80 °C temperature, they were plunged into liquid nitrogen. This was done quickly, as the cells deteriorate rapidly if temperature rises above –50 °C. When required, the vials were removed from the liquid nitrogen, thawed in warm water (37 °C) by holding the vial and vigorously shaking, and transferred into the complete medium previously equilibrated in a CO_2 incubator for 1 h.

2.10. The methylthiazolyldiphenyl-tetrazolium bromide (MTT) reduction assay

The MTT assay involves the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazan, an insoluble product, by mitochondrial reductase enzyme activity in living cells

Table. Primers for PCR.

Gene	Sequence	Annealing temperature	Base pairs	Accession number
oct-4	F- 5' CGCCCTATGACTTGTGTGGA 3' R- 5' TCGGCTCCAGCTTCTCCTT 3'	58	201	GU997625.1
c-kit	F- 5' CCAAACCTGAACACCGACAG 3' R- 5' CCATTCACCAGCCTGTCATG 3'	58	250	JN165090.1
bcl2	F- 5' GATGACTTCTCTCGGCGCTA 3' R- 5' GACCCCTCCGAACTCAAAGA 3'	62	165	AY547260.1
bcl2l1	F- 5' CAGGCGATGAGTTTGAACTG 3' R- 5' TCAGGAACCAGCGGTTGAAG 3'	62	369	NM001077486.2
bax	F- 5' GTGAGACCTCTAACCCCACC 3' R- 5' GGTCAGAGGTCATGAGGAGG 3'	62	175	GU731063.1
p53	F- 5' ACAACCTTCTGTCCTCCGAG 3' R- 5' GTTGCCAGGGTAGGTCTTCT 3'	62	197	FJ855223.1
β actin	F- 5' ACCCAGCACGATGAAGATCA 3' R- 5' GTAACGCAGCTAACAGTCCG 3'	62	187	U39357.1

(12). In the current study, after 24 h of cell culturing with different concentrations of vitamins based on experimental design in 96-well dishes (5000 cells per well), the viability of cells was determined using a kit (Thermo Fisher Scientific, Rockford, IL, USA) as per the manufacturer's protocol. Briefly, 12 mM MTT stock solution was prepared by adding 1 mL of sterile PBS to 5 mg of MTT. Then 10 µL of the 12 mM MTT stock solution was added to each well, which includes a negative control of 10 µL of the MTT stock solution added to 100 µL of medium alone, and incubated at 37 °C and in 5% CO_2 for 4 h. After that, 100 µL of the SDS-HCl solution (10 mL of 0.01 M HCl was added to 1 g of SDS) was added to each well and the microplate incubated at 37 °C overnight in a humidified chamber. The formazan concentration was determined by optical density at 570 nm.

2.11. Experimental design

First experiment: The effect of different concentrations of vitamin E and C alone and in combination on cryopreservation of SSCs

Experiment 1-1 The effect of different concentrations of vitamin E (12.5, 25, 50, and 100 µg/mL) and vitamin C (5, 10, 25, and 50 µg/mL) on viability of cryopreserved thawed SSCs

Experiment 1-2 The synergistic effect of effective concentration of vitamin E and C based on results of experiment 1-1 on the viability of cryopreserved thawed SSCs

Experiment 1-3 The expression of apoptotic related genes on cryopreserved thawed SSCs under effective concentration of vitamin E and C alone and in combination based on results of experiment 1-2

Second experiment: The effect of effective concentration of vitamin E and C alone or in combination based on results of experiment 1-3 on viability of transfected SSCs after cryopreservation and thawing

2.12. Statistical analysis

The data were analyzed with statistical software (SPSS 16, IBM, USA). One-way ANOVA followed by Duncan's multiple-range test was used for comparisons between multiple numeric datasets. The results were expressed as mean ± SEM and statistical significance was accepted at $P < 0.05$.

3. Results

The SSC suspension was obtained by enzymatic digestion two times. SSCs began to form colonies 7 days after culture and connections among colonies were visible on day 10 (Figure 1). *oct-4* and *c-kit* genes were detected as specific markers in these colonies (Figure 2) and they were positive for alkaline phosphatase staining (Figure 3).

3.1. First experiment: the effect of different concentrations of vitamin E and C alone and in combination on cryopreservation of SSCs

The first purpose of the present study was to evaluate the effectiveness of different concentrations of vitamin E and C alone or in combination on cryopreservation of ovine SSCs. Hence, the effective concentrations of vitamins under study were optimized based on the viability of cryopreserved and thawed cells by using the MTT assay. The results revealed that 12.5 and 25 µg/mL vitamin E and 5, 10, and 25 µg/mL vitamin C in cryopreservation medium significantly increased the viability of postthaw SSCs compared with the control ($P < 0.05$) (Figure 4). Then synergistic effects of effective concentrations were investigated by using MTT assay and expression of pro- and antiapoptotic genes. It was found that cryopreservation of SSCs in the presence of 25 µg/mL vitamin E and 10 µg/mL vitamin C could lead to increasing cell viability (Figure 5) and expression of antiapoptotic genes (*bcl2* and *bcl2l1*) (Figure 6).

3.2. Second experiment: the effect of vitamin E and C alone or in combination on viability of transfected SSCs after cryopreservation and thawing

The second purpose of the study was to explore whether the combination of vitamin E and C in basal freezing media improved the viability of postthaw transfected SSCs. As to the optimization of DNA and turbofect concentration, different concentrations of DNA (0.2, 0.4, and 0.8 µg) and turbofect (0.4 and 0.8 µL) in 100 µL of medium on Sertoli and SSCs were studied. The findings indicated that 0.8 µg of DNA and 0.4 µL of turbofect were the best combination for transfection of cells under investigation although SSCs had lower transfection efficiency than Sertoli cells (~30% vs. 70%, respectively) (Figures 7A and 7B) and only 10% of cryopreservation and thawing SSCs expressed GFP (Figure 7C).

To improve the efficiency of cryopreservation in transfected SSCs, as shown in the first experiment, 25 µg/mL vitamin E + 10 µg/mL vitamin C was used for 3 days on the culture medium and after that on cryopreservation medium. It was also concluded that by using a combination of vitamins, the efficiency of cryopreservation in transfected SSCs was improved in comparison with 12.5 µg/mL vitamin E, 5 µg/mL vitamin C, or SSCs after cryopreservation and thawing without vitamins (Figure 7F vs. 7D, 7E and 7C, respectively). The findings also demonstrated that by using a combination of vitamins (25 µg/mL vitamin E and 10 µg/mL vitamin C) the efficiency of cryopreservation in transfected SSCs was improved.

4. Discussion

In the present study, SSCs were harvested by enzymatic digestion twice and after enrichment cultured on Sertoli

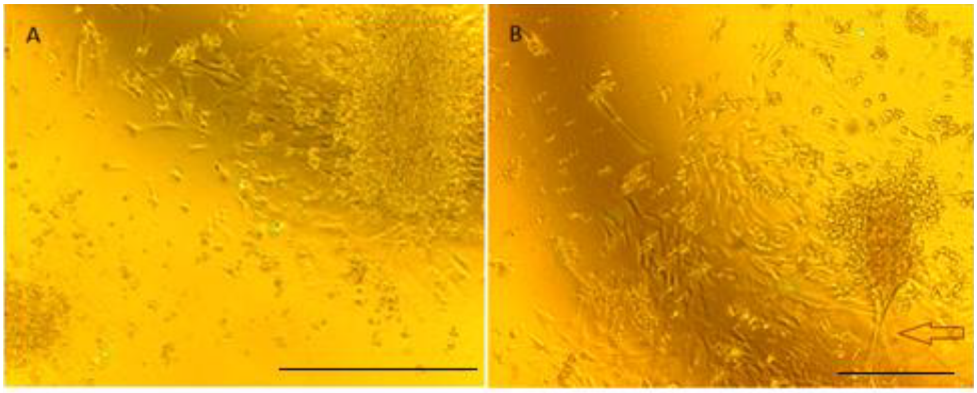

Figure 1. A) Formation of SSC colonies 7 days after culture and B) Connections among colonies were visible on day 10 (bar = 1 mm).

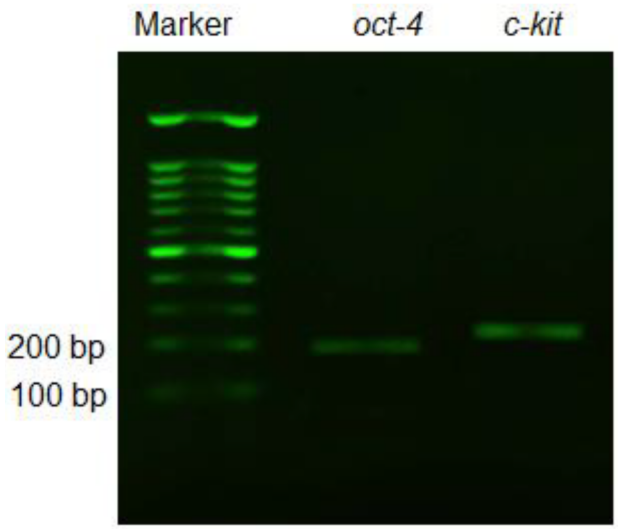

Figure 2. Expression of *oct-4* and *c-kit* genes in ovine SSCs.

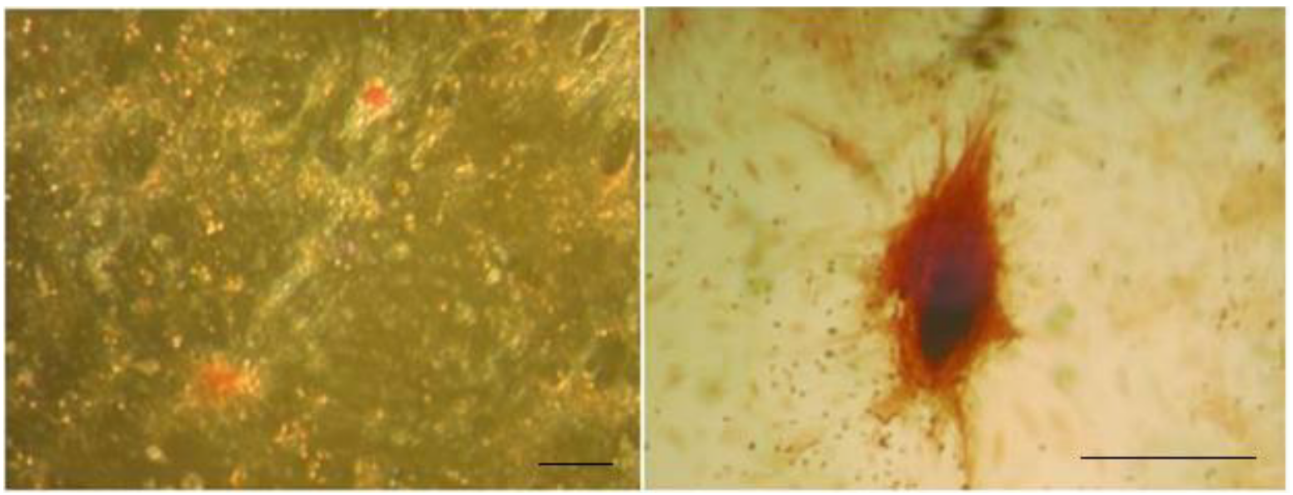

Figure 3. Alkaline phosphatase staining of ovine SSCs (bar = 1 mm).

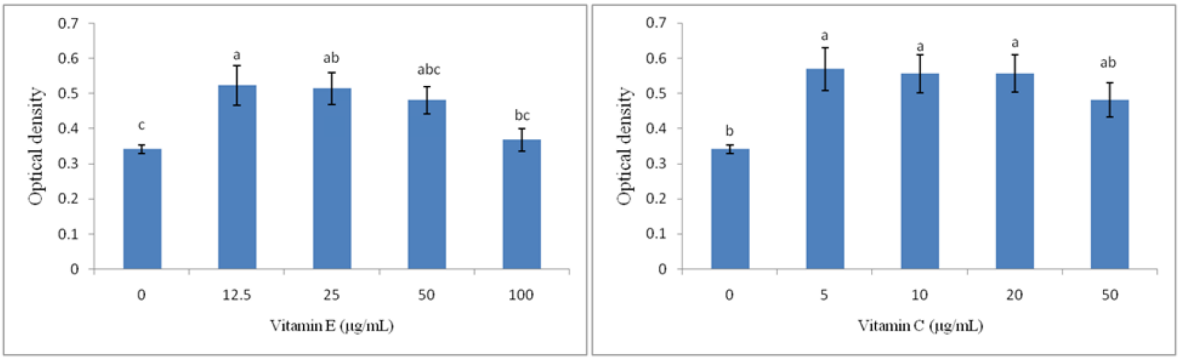

Figure 4. The effect of different concentrations of vitamin E and C on viability of cryopreserved thawed SSCs.

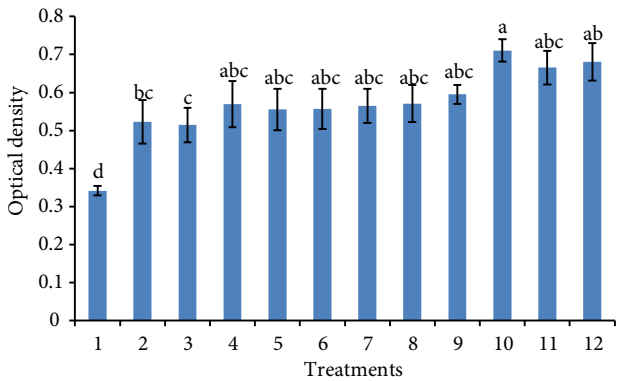

Figure 5. The synergistic effect of vitamin E and C on the viability of SSCs: 1) Control; 2) 12.5 µg/mL vitamin E; 3) 25 µg/mL vitamin E; 4) 5 µg/mL vitamin C; 5) 10 µg/mL vitamin C; 6) 25 µg/mL vitamin C; 7) 12.5 µg/mL vitamin E and 5 µg/mL vitamin C; 8) 25 µg/mL vitamin E and 5 µg/mL vitamin C; 9) 12.5 µg/mL vitamin E and 10 µg/mL vitamin C; 10) 25 µg/mL vitamin E and 10 µg/mL vitamin C; 11) 12.5 µg/mL vitamin E and 25 µg/mL vitamin C; 12) 25 µg/mL vitamin E and 25 µg/mL vitamin C.

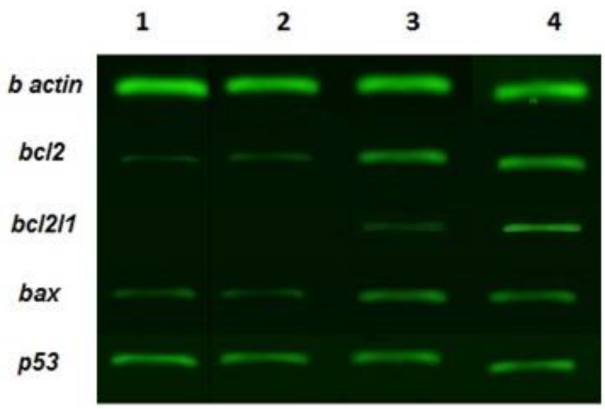

Figure 6. The expression of apoptotic related genes on cryopreserved thawed SSCs under different treatments: 1) Control; 2) 12.5 µg/mL vitamin E; 3) 5 µg/mL vitamin C; 4) 25 µg/mL vitamin E + 10 µg/mL vitamin C.

cells as a feeder layer. As reported by Wang et al. (6), in contrast to somatic testicular cells forming a confluent monolayer of cells, spermatogonia appeared in the single, paired, and clustered forms after 7 days of culture. Moreover, it was observed that connections among the cluster of SSCs were visible on day 10. The colony of SSCs

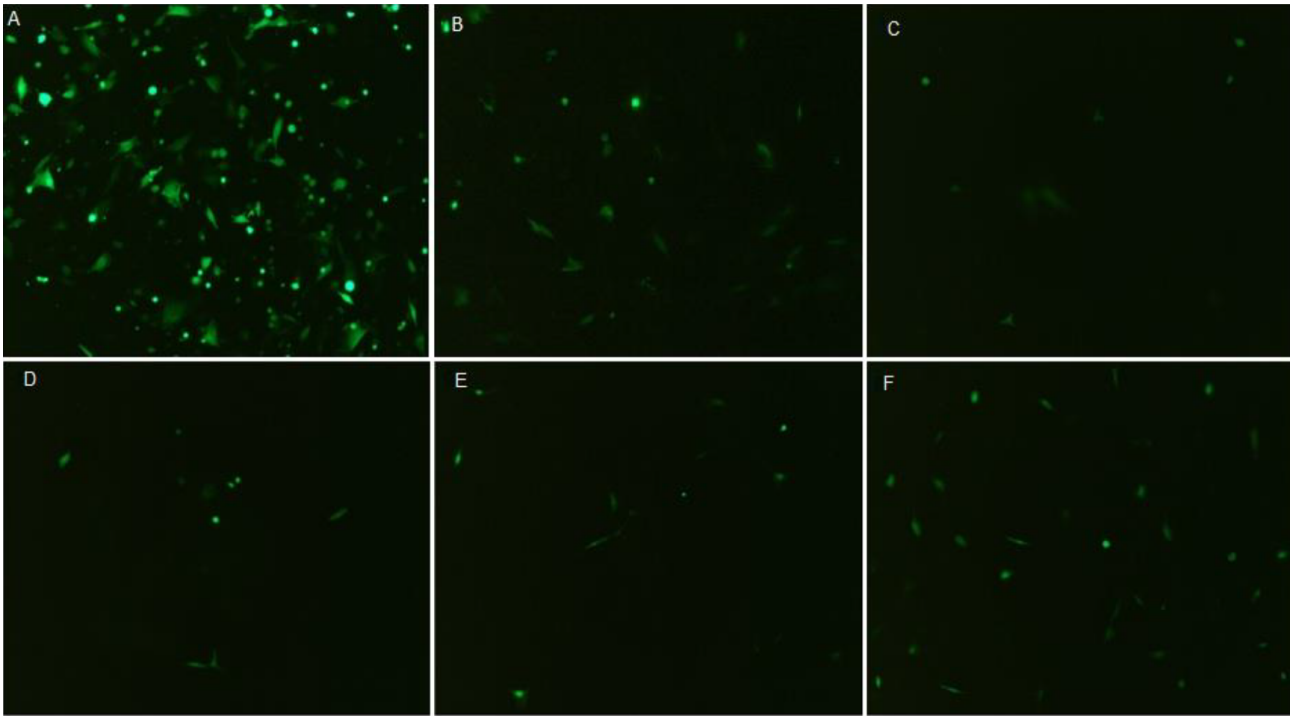

Figure 7. The effect of vitamin E and C on transfection efficiency of SSCs: A) Transfected Sertoli cells; B) Transfected SSCs before cryopreservation; C) Transfected SSCs after cryopreservation and thawing; D) Transfected SSCs after cryopreservation and thawing with 12.5 µg/mL vitamin E; E) Transfected SSCs after cryopreservation and thawing with 5 µg/mL vitamin C; F) Transfected SSCs after cryopreservation and thawing with 25 µg/mL vitamin E + 10 µg/mL vitamin C.

showed alkaline phosphatase activity and expression of ovine SSCs markers (*oct-4* and *c-kit*).

The cell culture and cryopreservation methods could be used for the maintenance of cell lines, but certainly cryopreservation has been the best method for long-term maintenance of SSCs because it can prevent aging and contamination, resulting in diminishing transformation in cell lines and preventing genetic changes in cell lines (8,13). However, the procedure does not come without limitations, one of which is the inability to retrieve adequate numbers of functional SSCs following freezing (8). This reduction in the survival rate of SSCs may be caused by the accumulation of toxic products of metabolism and ROS formation (9). To overcome these problems, different concentrations of vitamins E and C, alone and in combination, on cryopreservation medium of SSCs were evaluated. The results indicated that cryopreservation of SSCs in the presence of 25 µg/mL vitamin E and 10 µg/mL vitamin C can improve cell viability and expression of antiapoptotic genes (*bcl2*and *bcl2l1*). The findings reported optimal concentration and the synergistic effect of vitamin E and C on the freezing medium of ovine SSCs, which might have not been looked into in the previous literature, paving the way for the present research to produce innovative findings regarding the effectiveness of different concentrations of vitamin E and C as well as their combination on cryopreservation of SSCs and viability of transfected SSCs after cryopreservation and thawing.

Aliakbari et al. (8) analyzed *bax* and *bcl2* gene expression and reported that the apoptotic rate was decreased in the presence of antioxidants. α-Tocopherol, which is a lipid soluble antioxidant, can bring about increases in SSCs viability up to 70%–80%. By breaking the covalent links that ROS forms between fatty acid chains in lipid membranes, it can protect membrane components from the damaging effects of ROS (9). Aliakbari et al.'s (9) study clearly showed that, by using α-tocopherol, the apoptotic rates of cells were decreased. Proteins of the Bcl-2 family with either pro- or antiapoptotic activity have crucial roles in the regulation of apoptosis (14).

Wang et al. (6) stated that adding appropriate concentrations of vitamin C to SSC culture medium can promote stem cell proliferation, and its mechanism of action possibly involves regulation of ROS production and expression of apoptosis-related genes. Antioxidants have the ability to convert H_2O_2 to H_2O and O_2 and reduce the toxicity of ROS species as well as the deleterious effects of cryopreservation on cells (15).

Our findings demonstrated that the combination of vitamin E and C improved the viability of transfected SSCs after cryopreservation and thawing and the applied method of cryopreservation could increase cell viability and reduce

the expression of apoptosis-related genes. However, a lower amount of SSCs was transfected compared with Sertoli cells. Therefore, further research can be carried out regarding the improvement of transfected SSCs culture in vitro.

Acknowledgments

We would like to thank Dr Majed Masoumian and Amir Hossin Ahadi for their helpful comments during the research. This project supported by Iran National Science Foundation (Grant No. 93015593).

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