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Original article

CO-SUPPLEMENTATION OF FREEZING MEDIA WITH TREHALOSE AND VITAMIN C ON CELL VIABILITY AND APOPTOTIC GENE EXPRESSION IN OVINE SPERMATOGONIAL STEM CELLS

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Summary

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The purpose of this research was to investigate the trehalose and vitamin C (Vit C) co-supplementation of freezing media to create a successful cryopreservation protocol for conservation of ovine spermatogonial stem cells (SSCs). SSCs were cryopreserved and cultured with an essential freezing medium containing 200 mM trehalose, 40 µg/mL Vit C, and a combination of both for 3 weeks. Cell viability, colony number and diameter and mRNA levels of *Bax*, and *Bcl-2* genes were evaluated before and after cryopreservation with quantitative real-time PCR. The results showed that cells cryopreserved in freezing medium containing 200 mM of trehalose plus 40 µg/mL Vit C had considerably greater cell viability than the control group ($P<0.0001$). Up to the 3rd week of cell culture, supplementation of freezing medium with 200 mM trehalose resulted in significantly lower colonies diameters than in the control group. No significant differences were observed during the 1st to 2nd weeks in colony diameter and number of colonies between cells cryopreserved in the freezing medium containing either Vit C or trehalose compared with the control groups. Following thawing, the mRNA level of *Bax* in the Vit C + trehalose group was drastically lower than in those treated with trehalose or Vit C only ($P<0.0001$). High expression of *Bcl-2* in the 40 µg/mL Vit C group was recorded in the thawed cells compared to the control group ($P<0.0001$). These findings indicate that the concomitant use of antioxidants and sugar in the freezing medium can improve the quality and viability of SSCs after freezing via different mechanisms. Further studies are needed to clarify apoptosis and cell biomarkers in SSCs during freezing and thawing.

Key words: cryopreservation, spermatogonial stem cell, trehalose, vitamin C

INTRODUCTION

Spermatogonial stem cells (SSCs) have the natural ability to undergo differentiation and become mature spermatozoa or to regenerate to maintain sufficient residents

of SSCs for adult spermatogenesis (Dym, 1994). Male fertility relies on SSCs capable of self-renewing or differentiating into cells engaged in transformed spermatozoa and capable of handling genetic information up to the next generation (Kubota & Brinster, 2018). Due to the role of SSCs in the propagation of male germline and their susceptibility to death after chemotherapy for the treatment of cancer, significant effort has been made to improve techniques for their long-term survival (Dohle, 2010). These techniques include SSCs culture, SSCs transplantation, testicular tissue transplantation and cryopreservation (Sadri-Ardekani & Atala, 2014; Onofre *et al.,* 2020).

Cryopreservation is one of the main relative cultural approaches for the long-term survival of SSCs. Despite the benefit of cryopreservation, cellular redox disruption is caused by a rise of ROS content and, consequently, by oxidative stress. Excess cellular oxidant agents result in disruption of membranes, organelles, and biological molecules such as DNA, proteins, lipids, which can cause the activation of cell death. ROS plays an important role in apoptosis signalling processes in cell organelles, such as mitochondria regulated by *Bcl-2* family proteins (Redza-Dutordoir & Averill-Bates, 2016).

Typically, cryoprotective and apoptosis inhibitor agents are added to the freezing media alone or in combination with disaccharide to prevent cell damages (Sanssor *et al.,* 2003; Syvyk *et al.,* 2020). Sugars and antioxidants are examples of cryoprotective agents. Trehalose is a non-reducing disaccharide that prevents intracellular ice formation and crystallisation (Eroglu *et al.,* 2000). Previous studies have shown that the use of trehalose in freezing medium has contributed to improved cell viability, colonisation capability and viability of differentiated SSCs in mammals after freezing-thawing (Lee *et al.,* 2014; Aliakbari *et al*., 2016).

Vit C is a cofactor for Fe^{2+} and α-ketoglutarate-dependent dioxygenases, comprising a broad variety of different enzymes, such as collagen prolyl hydroxylase (vCPH) and epigenetic regulation of histone acetylation and DNA methylation (Cimmino *et al.,* 2018). Recent research has revealed that improving the culture media with adequate Vit C amounts can result in a more successful production of pluripotent stem cells in humans, animal somatic cells, and somatic cell nuclear transfer animal embryos (Chang *et al.,* 2011).

Despite the studies conducted on the role of sugars and antioxidants as cryoprotectants, there is no evidence of the simultaneous use of sugars and antioxidants in the freezing medium. The purpose of this research, therefore, was to investigate the co-supplementation of disaccharide and antioxidant-freezing media for cell viability and apoptosis-related gene expression in ovine spermatogonial stem cells.

MATERIALS AND METHODS

All substances and media used in this experiment were acquired from Sigma Company (ST. Louis, MO, USA) unless otherwise indicated.

Animal and testicular cell collection

In this experimental study, testes were obtained from six male lambs (two-month-old) using standard castration techniques. They were placed in Dulbecco phosphate buffered saline (DPBS), kept on ice for 1–2 hours, and transported to the laboratory. After initial removal of the tunica albuginea, testes were crushed into tiny pieces (around 1.75 g each testis) and

moved to a digestive solution containing 1 mg/mL of trypsin (Invitrogen), 1 mg/mL of collagenase IV, 1 mg/mL of hyaluronidase type II, and DNAse (5 µg/mL DMEM) in Dulbecco's modified Eagle medium (DMEM) and slowly shaken for approximately 1 hour. The distributed tissue was harvested and centrifuged at 1200 rpm for 3 minutes. In the second step digestion, the cell pellet was dissolved in DMEM containing 1 mg/mL of hyaluronidase, 1 mg/mL of collagenase IV (Gibco, USA) Type II, 5 µg/mL DNAse and incubated in a shaker at 37 °C for 30 minutes (Izadyar *et al.,* 2002).

Enrichment of SCCs population

The dispersed testicular cells were filtered through a 40 µm strainer. Single-cell suspensions were centrifuged at 500×g for 5 minutes at 25 °C and re-suspended in DMEM/F12 complemented by 2% foetal bovine serum (FBS). Then, the separated testicular cells were submitted to differential plating three times to purify SCCs (Zheng *et al.,* 2014). Briefly, the differential plating method was performed as followed: freshly isolated cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 for 2 hours in a full culture medium containing 5% CO_2, DMEM/F12 with 2% FBS, and then unattached cells were gathered and harvested in new dishes for another 6 hours. The unattached cells were picked again and replicated in fresh dishes for another 12 hours. The non-adherent cells were removed and used for *in vitro* culture.

Cryopreservation

SCC-enriched testicular cells were collected from culture three weeks after initial testis cell segregation and highly purified germ cells were accessed. The purity of germ cells was evaluated by immunocytochemistry staining (ICC) for octamer-binding transcription factor 4 (OCT4), a recognised marker for undifferentiated spermatogonia (Bahadorani *et al.,* 2011). In summary, after culture, single cells retrieved by trypsinisation were suspended in 0.5 mL of freezing media with 20% v/v dimethyl sulfoxide (DMSO), 60% v/v DMEM/F12, and 20% v/v FBS (GIBCO) was added gradually to cells (5×10^5) previously suspended in 0.5 mL DMEM/F12 with cryoprotective agents. This experiment had four groups: 1) control with the basic freezing medium, 2) basic freezing medium containing 200 mM trehalose, 3) basic freezing medium containing 40 µg/mL Vit C (Batch No: 224 Darou Pakhsh, Iran), and 4) basic freezing medium with 200 mM of trehalose + 40 µg/mL Vit C. Cryovials were placed in a cryoBox and stored at −80 °C for a minimum of one day. After that, vials were placed into liquid nitrogen for one-week.

Thawing process

Cryovials were removed from liquid nitrogen after a week, kept at room temperature, and then in a water bath at 37 °C for 2 minutes. The contents of cryovials were moved to a dish with a pre-warmed media (DMEM+10% FBS). The cells were rinsed and centrifuged at 3,200×*g* for 5 minutes (Jahnukainen *et al.,* 2007). After the supernatant was discarded, the pellet cells were used for several assessments, including cell viability, cell culture, and gene expression analysis (Izadyar *et al.,* 2002).

Cell viability

Cell viability was assessed with trypan blue dye before and after cryopreservation. Cell viability was measured as the percentage of non-stained cells in suspension compared with the total number of

cells counted in the chamber using a Neubauer haemocytometer. All cell counts were done in duplicate.

SSCs culture

SSCs were grown in all groups for three weeks after cryopreservation (a density of 5×10^5 cells/cm^2). DMEM supplemented by 10% FBS, 1× non-essential amino acid (Invitrogen, USA), 0.1 mM of 2-mercaptoethanol (Sigma, Germany), 10^3 U/mL of leukemia inhibitor factor (LIF; B&D, USA), 100 U/mL of penicillin, and 100 µg/mL of streptomycin (both Sigma, Germany), and 10 µg/ml of glial cell line-derived neurotrophic factor (GDNF; R&D, USA) was added to each well. The culture medium was replaced by new medium every two days. All cultures were stored in a suitable incubator at 37^{o}C, and sub-cultured using trypsin-EDTA every 7 days (Izadyar *et al.,* 2002).

Colony assay

After initial cell culture, the number and diameter of the colonies were evaluated for three consecutive weeks (7, 14, and 21 days after culture). The colony formation started earlier in treatment groups within 5 days, but this was detected in control within 7 days. All colonies in each dish were counted in duplicated independent experiments, diameters of colonies were obtained using an inverted microscope with an ocular grid, and images were processed using the Image J software (Koruji *et al.,* 2007).

Quantitative real-time PCR analysis

For the assessment of the best condition for SSCs cryopreservation, five SSC colonies from each group (containing three repeats) were randomly harvested immediately before and after cryopreservation. mRNA levels of *Bax* and *Bcl-2* were evaluated by real-time PCR (RT-PCR) using the primers shown in Table 1. Total RNA was isolated by chloroform and isopropanol and washed with 75% ethanol. cDNA was synthesised by a reverse transcription kit (Takara, China) with 1 µg of total RNA according to the manufacturer's information. The quality of cDNAs was evaluated by PCR analysis in comparison with *GAPDH* expression as a reference gene. Quantitative RT-PCR (RT-qPCR) was conducted in three different experiments for each sample using a SYBR Green PCR master mix package (Takara, Dalian, Chania). The melt curve test was conducted during each run to test the existence of non-specific PCR products and primer dimers. Relative mRNA expression for each gene was calculated

Table 1. Details of primer sequences, GenBank accession numbers, and expected product size of genes used for RT-PCR

Gene	Sequence 5′→3′	Amplicon size (bp)	Accession number
Bax	FW: CGACGGCAACTTCAACTGGG RV: CCCATGATGGTCCTGATCAACT	111	XM_012103831.2
Bcl-2	FW: CAGCTGCACCTGACGCCCTT RV: GATGCGCCCCCAGTTCACC	90	AF163774.1
GAPDH	FW: CCTGAGACAAGATGGTGAAGGT RV: CCTGAGACAAGATGGTGAAGGT	164	NM_001190390

GAPDH= glyceraldehyde-3-phosphate dehydrogenase; FW= forward; RV= reverse.

by the ratio of the target gene expression to that of references by Delta Delta CT analysis (Livak & Schmittgen, 2001).

Statistical analyses

All values are represented as mean ± standard deviation (SD). Data were analysed using a two-way analysis of variance (ANOVA), followed by post-hoc HSD using SPSS version 21.0. P value of less than <0.05 was considered to be significant. Each sample was tested in three independent experiments. The Graph Pad Prism software Version 8.0 was used for creating graphs.

RESULTS

Culture of SSCs

SSCs were isolated using a two-step enzymatic digestion protocol. Colonies were apparent after 4–7 days (Fig. 1). Formation of cell clusters in Vit C and trehalose+Vit C groups occurred on days 4 and 5, respectively, whereas this happened on day 7 in the control and trehalose groups.

Cell viability

The results showed that after 1 week, cells cryopreserved in the freezing medium containing trehalose+Vit C had considerably greater cell viability than controls ($P<0.0001$). During the 2nd to the 3rd weeks, no significant differences were found in cell viability between cells cryopreserved in the freezing medium containing trehalose+Vit C (Fig. 2).

Effects of vitamin C and trehalose on the number of colonies

After 1–3 weeks of cultivation, cells cryopreserved in the freezing medium containing trehalose+Vit C had a significantly higher number of colonies than the control group ($P<0.001$). During the 1st to

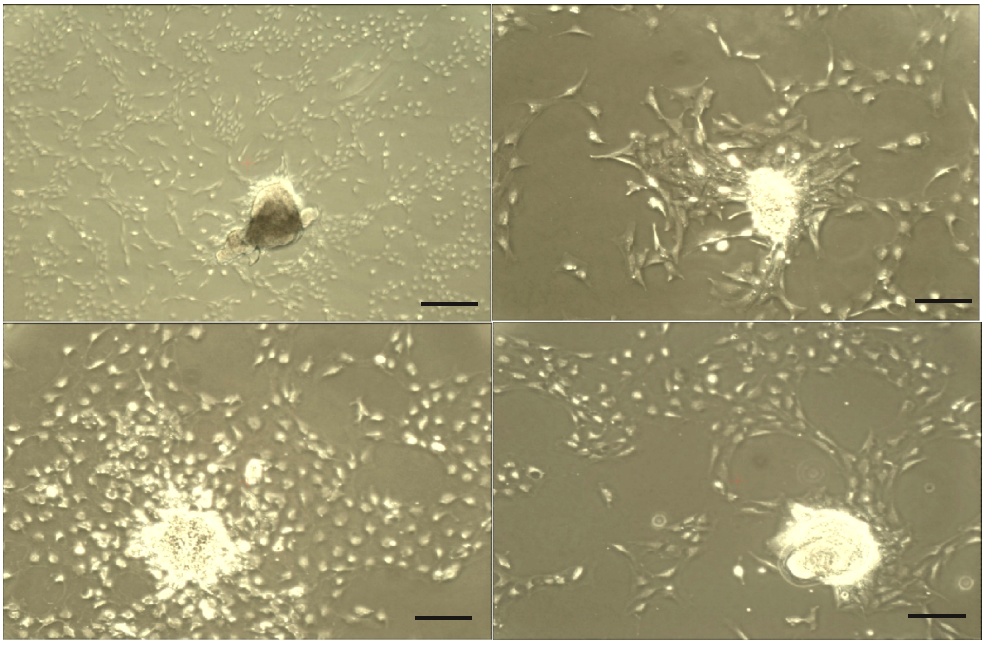

Fig. 1. Sheep spermatogonial stem cell colonies (scale bar=15 μm).

the 3rd weeks of cell culture, no significant differences were observed in the number of colonies between cells cryopreserved with freezing medium containing trehalose only or Vit C only compared with the control group. Accordingly, minimum and maximum numbers of colonies were present on the 1st and 3rd weeks, respectively (Fig. 3).

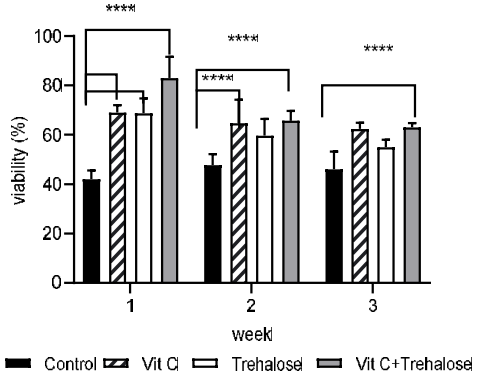

Fig. 2. Effect of vitamin C or/and trehalose on cell viability in ovine spermatogonial cultures (mean±SD of three independent experiments). *P<0.05; **P<0.01; ***P<0.001; ****P< 0.0001.

Effects of vitamin C and trehalose on colony diameter

As shown on Fig. 3, no significant differences were observed during the 1st to 2nd weeks in colony diameter between cells cryopreserved in the freezing medium containing 40 µg/mLVit C or 200 mM trehalose compared with the control group. During the 1st to the 3rd weeks of cell culture, supplementation of freezing medium with 200 mM trehalose resulted in colony diameters that were significantly lower than those in controls. After culturing for 3 weeks, cells cryopreserved in the freezing medium containing combined Vit C+ trehalose had a significantly higher colony diameter than those frozen with media containing either Vit C or trehalose only (P<0.0001).

Gene expression before and after freezing

The mRNA expression levels of *Bax* gene in the cell culture medium with 200 mM trehalose were significantly lower than those of the other groups. The mRNA expression levels of *Bax* was reduced for cells cryopreserved with Vit C+trehalose compared with 200 mM trehalose or 40

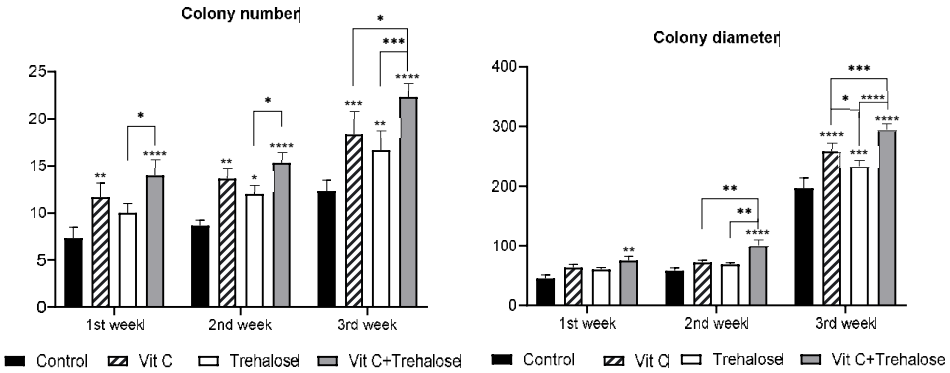

Fig. 3. Effect of vitamin C or/and trehalose on colony number and colony diameter (µm) in ovine spermatogonial cultures (mean±SD of three independent experiments);
* P<0.05; ** P<0.01; ***P<0.001 ****P< 0.0001.

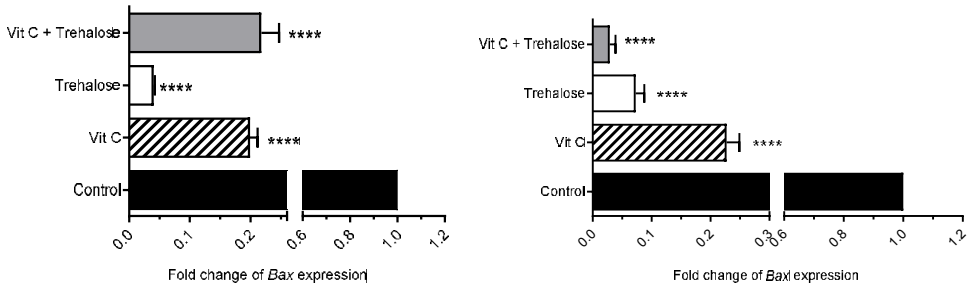

Fig. 4. Expression pattern of *Bax* genes in spermatogonial stem cells colonies in the three experimental groups before (left) and after cryopreservation (right). Values are mean±SD of three independent experiments; **** P< 0.0001.

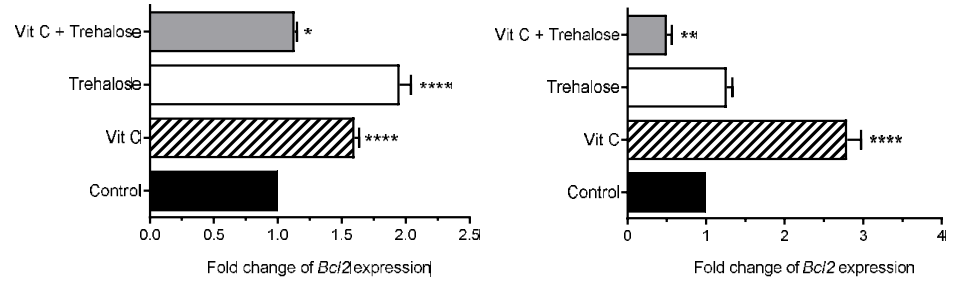

Fig. 5. Expression pattern of *Bcl-2* genes in spermatogonial stem cells colonies in the three experimental groups before (left) and after cryopreservation (right). Values are mean±SD of three independent experiments; *P<0.05; **P<0.01; ****P<0.0001.

µg/mL Vit C (P<0.0001) (Fig. 4). The results showed that *Bcl-2* mRNA expression in the cell culture medium with 200 mm trehalose was greater than that in the control group (P<0.0001). Interestingly, the addition of Vit C and trehalose to cell culture medium had a less pronounced effect on Bcl-2 mRNA expression (Fig. 5).

SSCs cells cryopreserved in freezing medium containing 40 µg/mL Vit C had significantly (P<0.0001) increased *Bcl-2* gene expression compared with the control group (Fig. 5). However, addition of 200 mM trehalose to the freezing medium did not seem to have an effect on *Bcl-2* mRNA expression after freezing compared with the control group.

DISCUSSION

Optimal cryopreservation of SSCs is crucial for the success of fertility restoration. The purpose of this research was to find an appropriate protocol to prevent SSCs damage after freezing-thawing. The obtained data indicated that the addition of antioxidants and sugars in the freezing medium could be useful for the cryopreservation due to the increased cell viability and reduce cryodamage. Consistent with our results, Koruji *et al*. (2007) found that adding antioxidants to a freezing medium improved cell viability. Besides, the inclusion of trehalose in the freezing media was confirmed to be useful for the post-thaw survival of enriched germ cells in SSCs (Lee *et al.,* 2013).

Consistent with these studies, cryopreservation of different types of cells, such as mammalian germ cells and other stem cells, in a medium containing 200 mM trehalose could improve viability rate (Eroglu *et al.,* 2009; Ha *et al.,* 2016). It has been shown that trehalose, as a non-reducing disaccharide, can help to stabilise proteins within the plasma membrane by creating chemical bonds with cell membrane phospholipids during the freezing cycle by formation of hydrogen bonds with the respective proteins and phospholipids, leading to increased freezing tolerance in SSCs (Eroglu *et al.,* 2000; Ha *et al.,* 2016). Besides, previous studies showed that low-dose trehalose resulted in increased cell viability and propagation rate compared to the DMSO group (Lee *et al.,* 2014). These data indicated that cell viability could be dependent on the dose and duration of cell culture. In addition, the obtained result data demonstrated that supplementation of freezing medium with trehalose+Vit C improved the number and diameters of colonies. Physical contact and secreted growth factors and cytokines influenced the colonisation of SSCs and supported SSCs proliferation and colonies formation (Kourji *et al.,* 2007). However, in 50 Mm trehalose-treated groups, the cell viability was higher after thawing while a study of Lee *et al.* (2013) has shown that the treatment of SSCs with 200 mM trehalose resulted in higher proliferation capacity of colonies which was persistent up to 3rd week after thawing step.

Our results showed that adding Vit C to the freezing medium had not a much greater effect on the number of colonies and survival after thawing. This study was not in full agreement with previous research, which demonstrated that adding Vit C to the SSC culture medium could improve proliferation (Wang *et al.,* 2014). Different results may be due to the type of compound and different doses of Vit C in the freezing medium (Huang *et al.,* 2011; Hu *et al.,* 2012).

The previous results demonstrated that the cryopreservation of sperm with disaccharides and antioxidants might decrease the apoptosis rate during freezing (Lee *et al.,* 2014; Aliakbari *et al.,* 2016; Ha *et al.,* 2016). Our results showed that co-supplementation of trehalose and Vit C to the freezing medium led to considerably down-regulated *Bax* gene expression after thawing. Consistent with these studies, adding a high dose of trehalose to freezing media led to increased proliferation and decreased percentage of apoptotic cells (Eroglu *et al.,* 2009). It was reported that supplementation of 100 mM and 200 mM of trehalose to the freezing medium resulted in a relatively lower percentage of apoptotic cells after thawing (Lee *et al.,* 2013). The obtained results revealed the mRNA levels of *Bcl-2* gene had increased in freezing medium containing 40 µg/mL Vit C. In agreement with our results, adding Vit C to the freezing medium resulted in decrease the mRNA levels of *Bcl-2* gene in other studies (Wang *et al.,* 2014; Shabani *et al.,* 2017). Previous studies stated that ROS production was blocked and the apoptosis signalling pathway was regulated in the presence of suitable Vit C doses (Hu *et al.,* 2012). Studies also established a possible mediating role of antioxidants in the expression of many factors involved in cell apoptosis, such as Bcl2 and caspase 3 (Amidi *et al.,* 2019). These findings might offer new insights into the potential principal mechanisms of the cryopreservation and anti-apoptosis effects of trehalose and Vit C through presumably different mechanisms. Based on the findings of this research, the protecting mechanism of adding trehalose is

probably due to its stabilising effects on cell membrane, therefore minimising the osmotic changes during the freezing and thawing process (Wu et *al.,* 2005). However, Vit C functions as a growth promoter improving cell proliferation, DNA synthesis and it can protect cells against the detrimental effects of ROS (Wang *et al.,* 2014).

In conclusion, the concomitant use of antioxidants and sugar in the freezing medium can improve the quality and viability of SSCs after freezing by different mechanisms.

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