IJAS Iranian Journal of Applied Animal Science

Improving the Cryostorage Parameters of Ram Germ Stem Cells by Supplementing with Soybean Lecithin as a Cryoprotectant

Research Article

S. Ghahremani[1], M. Sharafi[1*], B. Ghasemi Panahi[2] and A. Javanmard[2]

[1] Department of Animal Science, Faculty of Agriculture, University of Tarbiat Modares, Tehran, Iran
[2] Department of Animal Science, Faculty of Agriculture, University of Tabriz, Tabriz, Iran

Received on: 31 Mar 2024
Revised on: 27 Sep 2024
Accepted on: 15 Nov 2024
Online Published on: Dec 2024

*Correspondence E-mail: sharafi2000@gmail.com
© 2010 Copyright by Islamic Azad University, Rasht Branch, Rasht, Iran
Online version is available on: www.ijas.ir

ABSTRACT

To date, the challenging problem of reducing cryodamage caused by the freezing and thawing process of spermatogonial stem cells (SSCs) is still at the experimental stage, and understanding the complexity of this puzzle is crucial. More recently, soybean lecithin (SL) has been claimed to be a suitable molecule to protect SSCs from cold shock throughout the cryostorage process. Based on this motivation, the main aim of the present research is to evaluate the cryostorage parameters of ram germinal stem cells by SL supplementation as a cryoprotectant agent. For this purpose, SSCs were isolated from the testicular tissue of a 2-month-old ram and we then added different concentrations of SL (control, 0.5, 1 and 1.5 percent) and then added them to the basic media described. Next, cryostorage parameters (cell viability, number and area of colonies) were assessed per treatment. To examine this statistically, we calculated a one-way ANOVA and HSD test with SAS and a $P < 0.05$ was considered significant. Interestingly, our results showed that cell viability and colony number and area were significantly higher during supplementation with 0.5% SL than the other treatments ($P<0.05$). However, SSCs preserved in 1.5% SL have adverse effects due to their toxicity rate. On this basis, our current findings demonstrate an improvement in cryostorage parameters of ram germ cells by supplementation with SL. This assumption could be addressed in future studies.

KEY WORDS cryopreservation, cryoprotectants, Ghezel sheep, soybean lecithin, spermatogonial stem cells.

INTRODUCTION

From a practical perspective, transplantation of sperm-producing stem cells from desirable rams to replace male surrogate mothers offers a unique approach to preserving and manipulating germplasm in sheep production (Zhao *et al*. 2021). In general, SSCs are defined as colonogenic cells capable of both self-renewal and differentiation (Ibtisham *et al*. 2020). The reason why SSCs are different from other cells is that they can pass on their genetic information to future generations, but these cells only make up a small percentage (0.03%) of all germ cells in the testis (Kim *et al*. 2015; Ibtisham and Honaramooz, 2020).

Typically, cryopreservation of SSCs is an important process because it can support the high genetic elite of spermatogenesis through SSCs transplantation technology. However, establishing efficient cryopreservation is not easy, and in this process stem cells are often damaged, reducing their value for experiments and treatment. The ability to successfully cryopreserve SSCs has opened a route for long-term storage of these cells without apparent impairment of their function (Feng *et al*. 2020). The process of cell freezing is a beneficial technique for preserving SSCs. However, due to fluctuations in osmotic pressure, the cryopreservation method can cause damage to cell membranes and biological changes that ultimately affect cell

fertility (Pirnia *et al*. 2020). To address this problem, numerous cryoprotectants have been used to minimize post-freezing injuries. However, the use of an appropriate preservative to minimize the effects of freezing is still debated. A review of previous research shows that several attempts to improve basic cryopreservation media by slow freezing is a superior method because it avoids the formation of intracellular and extracellular ice and biologically deleterious effects (Kazemzadeh *et al*. 2022). A number of authors have found that cryoprotectants containing low-density lipoproteins (LDL) have the ability to trigger a gelation process when frozen and thawed. By forming a protective layer on the surface of the sperm membrane, LDL can reduce crystal formation and damage to the membrane and increase membrane flexibility against cryoinjury (Chelucci *et al*. 2015; Mehdipour *et al*. 2018; Shamsollahi *et al*. 2018). To date, many studies are still needed to reduce the cryodamage caused by the freezing and thawing process of SSCs, and it is crucial to understand the complexity of this puzzle. More recently, SL has been claimed to be a suitable molecule to protect SSCs from cold shock during the cryostorage process (Wang *et al*. 2014).

SL of plant origin is a combination of phospholipid and various fatty acids such as oleic acid, palmitic acid, arachidonic acid, etc., which has been evaluated in several studies as a cryoprotectant in sperm freezing (Sharafi *et al*. 2015; Miguel-Jimenez *et al*. 2020). Several studies have shown that *SL*, by lowering the freezing point and replacing membrane phospholipids, leads to the formation of a protective layer around the sperm membrane, which can protect the membrane from cold shock. However, more information is required (Mehdipour *et al*. 2018). It is crucial to add an optimal amount of intracellular and extracellular cryoprotectants to the freezing medium (El-Shahat and Hammam, 2018). Dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO, USA) as a base medium is one of the most common cryoprotectants that can reduce or prevent cell damage and ice formation upon freezing. SL, a non-permeable cryoprotectant, may also contribute to cell membrane integrity (Motta *et al*. 2014; Mehdipour *et al*. 2018).

To shed light on this new area, the purpose of the current study was to determine an optimal SL value for the freezing media and obtain more information about the protection of Ghezel lamb SSCs. To our knowledge, this is the first report and particularly not a study to evaluate SL in terms of various parameters, including assessment of viability rate and post-thaw colonization of ram SSCs.

MATERIALS AND METHODS

Chemicals

Unless otherwise noted, chemicals were purchased from Sigma Co (St. Louis, MO, USA). In addition, the SL (SL; Sigma Aldrich, St. Louis, USA) used in this experiment had the following chemical formula: P3644 Sigma L-α-Phosphatidylcholine - from soybean, type IV-S, ≥30% (enzymatic) (Figures 1).

Animals

Male lambs (2 months old, n=3) were maintained under standard conditions. All experiments in this study were approved by the University Ethics Committee and conducted in accordance with university guidelines (Approval number: IR.MODARES.REC.1399.246).

Sampling

In this research, SSCs were obtained from two-month-old male lambs (Ghezel breed). Lambs were locally anesthetized with 0.3 mg/kg IM administration of xylazine. In addition, each lamb received a local injection of 3 ml with 2% lidocaine 30 minutes before surgery. All vital signs such as respiration and heart rate were checked postoperatively, and the lambs were also administered flunixin meglumine (2.2 mg/kg body weight, IM) for three days (Qasemi-Panahi *et al*. 2018).

The testicular sperm extraction (TESE) technique was performed to obtain samples from animals (Mohebbi *et al*. 2021). Tunica albuginea and seminiferous tubules were seen after incision of the right seminiferous membrane. Samples were placed in Dulbecco's Modified Essential Medium high in glucose (DMEM; Sigma, St. Louis, MO, USA) and kept on ice for 1–2 hours during transport.

Cell isolation and culture

Under sterile conditions, tunica vaginalis and tunica albuginea were carefully removed, then samples were washed with phosphate buffer saline (PBS, Sigma Aldrich, St. Louis, USA) and minced into small pieces. After mechanical digestion, a two-step enzymatic disruption was used to prepare single-cell suspensions from testicular tissue (Aliakbari *et al*. 2017). In the first step of the enzymatic digestion, pieces of testicles were suspended in DMEM consisting of collagenase type IV (1 mg/mL; Sigma) and trypsin (1 mg/mL; Sigma) and incubated at 37 °C for 40 min. In this step, after washing three times in DMEM medium, most of the interstitial cells were removed and the seminiferous tubules were separated. Thus, the fragments of the seminiferous tubules were sent to the 2nd step of enzymatic processing. In the second step, seminiferous cord fragments were incubated with the same enzymes as described above for 45 min and then enzymes were inactivated by adding 10% fetal bovine serum (FBS, Sigma Aldrich, St. Louis, USA). The obtained cell suspension was centrifuged to obtain reasonable pellets (1500 rpm for 5 min), then the digested cells at this time were washed with PBS.

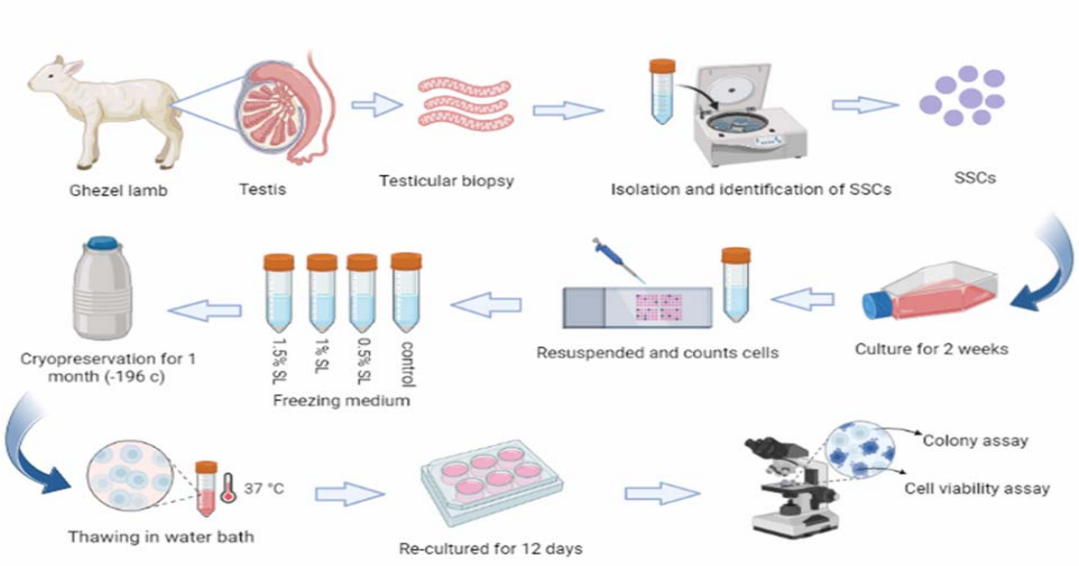

Figure 1 Schematic experimental design of the cryopreservation process in Ghezel lamb spermatogonial stem cells to evaluate the effects of soybean lecithin (SL) on cell viability, number and area of colonies

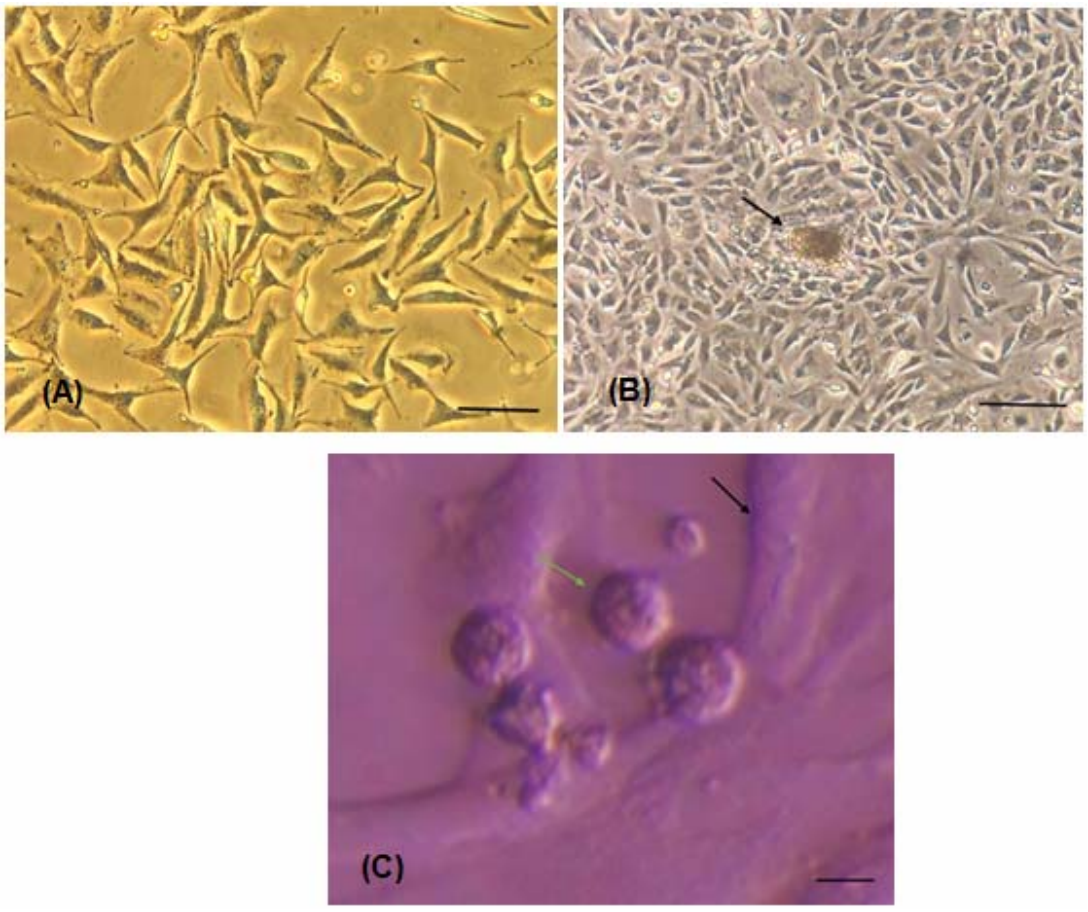

Figure 2 (A) Sheep Sertoli cells. Scale bar 100 µm (400×). (B) Sheep spermatogonial colony and Sertoli cells (co-culture). Scale bar 150 µm (100×). (C) Spermatogonial (green arrow) and Sertoli cell (black arrow) in sheep (co-culture). Scale bar 15 µm (400×)

Finally, the cell suspension was filtered through a nylon mesh cell strainer (pore size 0.22 µm; Biofilm, China) and treated with culture medium consisting of 90% DMEM, 10% FBS, 1% antibiotic (100 IU/mL penicillin, 100 mg/mL streptomycin) and then placed in a 25 cm^2 cell culture flask and incubated at 37 °C in a humidified atmosphere of 5% CO_2 (Figures 2). The culture medium was changed twice a week and passaged 1:2 or 1:3.

Immunocytochemistry for confirmation of SSCs

Colonies obtained from SSCs were immunocytochemically stained with anti-Oct-4. Specifically, anti-Oct-4 (Abcam) diluted in TBS/BSA was used for the slides. After one hour, the slides were washed. After addition of FITC-conjugated donkey polyclonal secondary antibody to goat IgG, further incubation was continued for 45 minutes at room temperature. Washes were performed and fixed in PBS-glycerol 90% and examined under a fluorescence microscope (Olympus, Tokyo, Japan). In addition to size and morphology, identification of spermatogonial cells was carried out by Oct-4 tracing in the colony cells. Spermatogonial-derived colonies showed Oct-4 expression (Figures 3). This finding is in agreement with reports of Jeong *et al*. (2003), Kubota *et al*. (2004), Shi *et al*. (2006), and Qasemi-Panahi *et al*. (2018) who verified Oct-4 expression in the SSCs.

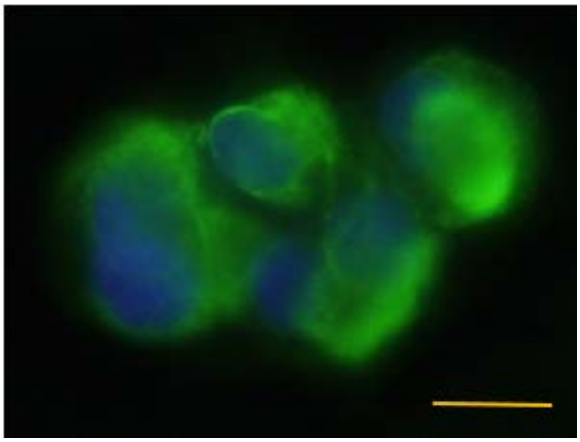

Figure 3 Oct-4 immunocytochemical staining of ram spermatogonial stem cells. Oct-4-positive colony. Nuclei were counterstained with DAPI. Scale bar 15 µm

Cryopreservation

The SSCs collected from the enzymatic digestion of the testis tissue were co-cultured on the Sertoli cells for 2 weeks. The cells obtained were randomly divided into 4 groups and the experiments were duplicated for each of the 4 groups. Four different freezing media were slowly added to the cell suspension. The basic freezing medium (1): control group based on DMEM supplemented with 10% DMSO, 70% FBS (Qasemi-Panahi *et al*. 2018), (2): 0.5% SL; (3): 1% SL, (4): 1.5% SL were added to the basic freezing media. It is striking that the specified percentages of *SL* were completely dissolved in 20% DMEM using a vortex mixer and then filtered through a 0.22 µm filter. In our experiment, manual freezing method was performed slowly with noncontrolled-rate freezing (Izadyar *et al*. 2002). All cryovials (Biofilm, China) were placed in an insulated container (polystyrene) in a -4 °C refrigerator for 2 hours and then stored in a -80 °C freezer for at least 24 hours. After overnight storage, cryovials consisting of frozen cells were held over liquid nitrogen for 3 minutes and finally immersed in a liquid nitrogen tank for 1 month (Qasemi-Panahi *et al*. 2018).

Thawing procedure

After removal from liquid nitrogen, frozen cells were thawed by immersion in a water bath at 38 °C for 2 min (Mirzapour *et al*. 2013). The cryovial contents of the vials were transferred to a new Falcon and diluted by the addition of DMEM + 10% FBS. Then the diluted compound was washed twice with medium and centrifuged at 1200 rpm for 5 min (Aliakbari *et al*. 2016). The supernatant was drained, then the cell pellet was resuspended with DMEM + 10% FBS and cultured for 12 days in 3 cm^2 plates at 37 °C and 5% CO_2. Cell viability was analyzed after thawing.

Colony viability assay

The cell viability analysis was measured with a hemocytometer and evaluated immediately after thawing. The number of spermatogonial cells was determined by the staining exclusion method (0.04% trypan blue solution): viable cells were not stained by trypan blue; however, dead cells were stained blue (Strober, 1997). The total number of dead cells and live cells were examined by light microscopy after cryopreservation. Furthermore, all treatments were repeated three times.

Colony assay

Determination of the number of colonies derived from SSCs was started after 5 days of recultivation and was performed every 3 days after the appearance of colonies during culture (6^{th}, 9^{th} and 12^{th} day). This assessment was measured with an inverted microscope (Nikon, Japan) equipped with an eyepiece grid. In addition, on these days, areas of colonies cultured with different percentages of SL were examined with the same microscope (this was repeated three times).

Statistical analysis

Results and statistical analyzes were performed in a fully randomized design using SAS System software (SAS, 2013). One-way ANOVA and HSD test were used to test

for differences between treatment groups. A p-value of <0.05 was considered significant. In this study, the following statistical model was used for data analysis:

$Y_{ij}= \mu + T_i + e_{ij}$

Y_{ij}: the j^{th} observation of the i^{th} treatment.
μ: mean of observations.
T_i: treatment effect.
e_{ij}: experimental error.

RESULTS AND DISCUSSION

The effects of SL on frozen and thawed sheep SSCs were examined. Identification of spermatogonial cells was done by tracking on Oct-4 in the colony cells. Spermatogonial-derived colonies showed Oct-4 expression (Figure 3). Since our team has been researching these cells since 2010, we have checked various markers, e.g., Oct-4 (Mohebbi *et al.* 2021) and PLZF (Qasemi-Panahi, *et al.* 2018) on these cells. Since our aim was to improve the colonization and growth of spermatogonial stem cells in the co-culture system, we reported the average total area and number of colonies after the freeze-thaw process (Tables 1 and 2).

Table 1 Comparison of colony number between control and experimental groups**.** Results from three independent experiments were used for each group

Groups	Time (days)			TANC[1]
	6	9	12	
Control	20[b]	24[b]	29.33[b]	24.45[b]
0.5% SL	47[a]	54.67[a]	69.67[a]	57.11[a]
1% SL	18.67[b]	23[b]	27.67[b]	23.11[b]
1.5% SL	9[b]	11.67[b]	14.67[b]	11.78[b]
P-value	0.0004	0.0006	0.0002	0.0001
SEM	3.64	4.32	4.64	3.03

TANC: total average number of colonies and SL: soybean lecithin.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 2 Comparison of colony area (µm) between control and experimental groups. Results from three independent experiments were used for each group

Groups	Time (days)			TAAC
	6	9	12	
Control	205.77[b]	212.37[b]	225.78[b]	214.64[b]
0.5% SL	402.67[a]	410.48[a]	425.85[a]	413.00[a]
1% SL	200.26[b]	210.33[b]	221.45[b]	210.68[b]
1.5% SL	133.36[b]	142.06[b]	151.40[b]	142.27[c]
P-value	0.0001	0.0001	0.0001	0.0001
SEM	22.08	17.42	22.30	11.77

TANC: total average number of colonies and SL: soybean lecithin.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

After 2 weeks of co-culture with Sertoli cells, the majority of cells (95%) were viable prior to freezing. The viability of the cryopreserved cells was assessed by trypan blue staining. Data on the effect of *SL* in freezing medium on the viability of SSCs are presented in Figure 4. The effect of SL on cell viability was evaluated and the result of the present study showed that the percentage of viability of cryopreserved cells in the presence of 0.5% SL (80%) was significantly (P<0.05) higher than those with 1% (60%) and 1.5% SL (46.67%) treated cells and controls (63%). Among the various conditions measured in this study, the best results were obtained with 0.5% SL. However, the consequences for the 1.5% SL freezing media were insignificant compared to the other groups described above, so the addition of 1.5% SL did not significantly affect viability.

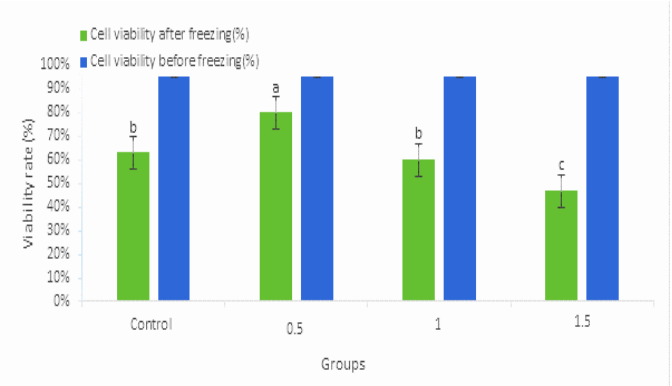

Figure 4 Comparison of cell viability between control and experimental groups before and after freezing. Bars with different letters show significant differences (P<0.05) between different treatment groups, while the same letters represent a lack of differences between columns. Results from three independent experiments were used for each group

The data on the effect of SL in freezing medium on the colony number of SSCs at various percentages are presented in Table 1. As shown in Table 1, our results showed that the number of colonies in the 0.5% SL group was significantly higher than the other groups at 12 days (P<0.05).

SL was an excellent cryoprotectant and the highest colony number was 69.67. Therefore, 0.5% SL could significantly protect Ghezel lam SSCs. In addition, the number of colonies in the control group was higher than the number in 1% and 1.5% SL, but there was no significant difference (P>0.05).

Cell membrane damage was reduced during freezing in the presence of 0.5% SL, and even the number of colonies increased during 12 days of co-culture with Sertoli cells. The mean total number of spermatogonial colonies in each experimental group was assessed, and the data are presented in Table 1.

In addition, the highest mean total number of colonies was observed in the 0.5% SL group, and there were statistically significant differences between these treatments and other groups (P<0.05).

As shown in Figure 5(A) the colonies' number in all groups gradually increased during the 12-day co-cultivation. In addition, the highest number of colonies refers to the 12th day of co-culture with Sertoli cells after cryopreservation.

As shown in Table 2, the result of our study represents that the area of colonies (µm) after 12 days was larger in the 0.5% SL treated groups than in other groups, and the differences were significant (P<0.05). Also, the colony in the control group was better than the other treatment groups, but there was no significant difference (P>0.05).

The result of the effect of SL on colonies' area of Ghezel sheep SSCs at different percentages is shown in Table 2. The 0.5% SL data was chosen as the best colony area during 12-day co-cultivation of freeze-thawed SSCs with Sertoli cells. In addition, the area of colonies in the 1% SL group was not significantly different from the control group (P>0.05).

The effects of different experimental groups on colony area at days 6, 9 and 12 are shown in Figure 5(B) The highest colony area relates to the 0.5% SL group (P<0.05) on day 12 of co-culture and also the lowest area relates to the 1.5% SL treated group on day 6 of co-culture (P<0.05). The area of the colonies has gradually increased these days.

In vitro proliferation and preservation of SSCs has many potential applications in reproductive science and also offers the possibility of passing on genes to future generations (Ibtisham *et al*. 2021). The aim of this study is to shed new light by investigating the cryoprotective effect of different SL percentages on SSCs from frozen and thawed Ghezel lambs. Therefore, this research project attempted for the first time to determine the best SL dose and evaluate cell viability and colony number and area during co-culture after the freeze-thaw process. For this purpose, the testicular cells of 2-month-old Ghezel lambs were used to obtain corresponding populations of spermatogonial and Sertoli cells. It appears that 2-month-old lambs are the right age to isolate these cells, and these testes have proven to be the best source. Similarly, this view is supported by Zargarzadeh (Zargarzadeh *et al*. 2019).

Cryopreservation of SSCs occupies a prominent place to study male fertility, long-term protection of fertilizing cells, factors involved in the development of SSCs, etc. Therefore, the cryopreservation method should be optimized for applications of SSCs in assisted reproductive technologies (Aliakbari *et al*. 2016). However, there are only limited studies on the effects of different cryoprotectants on sheep SSCs.

In the present study, to achieve a more efficient cryopreservation medium, a different percentage of SL was used as a non-permeable cryoprotectant to preserve Ghezel lamb SSCs from the freeze-thaw process. The viability of SSCs after the freeze-thaw process is critical for clinical applications. After thawing, the results showed that the addition of 0.5% SL to the freezing medium significantly (P<0.05) increased the viability of SSCs, in addition to proliferation in the culture after thawing and colonization. Therefore, 0.5% SL provided better cryoprotection for these cells. In line with previous studies, the beneficial effect of SL on its cryoprotective abilities on sperm of different species has been reported in many studies (Sharafi *et al*. 2015; Dalmazzo *et al*. 2018; Mousavi *et al*. 2019; Sun *et al*. 2021). However, there is little information on the preservative ability of SSCs by SL. Some unknown protective mechanisms have been proposed and it has also been concluded that there are similarities between spermatogonial cells and sperm. According to pervious published reports, the combination of this freezing medium helps to stabilize cell membranes by cold shock, and also the main component of SL is low-density lipoprotein, so external phospholipids could cover the membrane of spermatogonial cells during cryopreservation (Ustuner *et al*. 2014). The preserving function of *SL* during the freeze-thaw process is taken over by phospholipids. The first mechanism for the cryoprotective effect of SL is that phospholipids, by reducing the exchange of plasmalogens, can lead to a lowering of the freezing point of ice crystal formation, thereby reducing mechanical damage to the membrane (Fathi *et al*. 2019; Sun *et al*. 2021). The second protective mechanism of SL was suggested that phospholipids from SL could combine with sperm membrane to form a preservative film instead of forming deadly intracellular ice crystals, therefore these phospholipids could replace some phospholipids of spermatogonial cell membrane and lead to cell membrane resistance (Salmani *et al*. 2014; Wang *et al*. 2014).

In our experiment we found that the optimal level of SL in the freezing medium is 0.5% because this medium contains an intracellular compound (DMSO) that prevents the formation of ice crystals in the cells and an extracellular cryoprotectant (SL), which stabilizes the cell membranes. There were no significant differences between the 1% SL and the control group (P>0.05).

It contributed to the low viability of SSCs due to the absence of SL in the control group. On the other hand, we observed a decrease in viability in the freezing medium containing 1 and 1.5% SL. The percentage of SL added to the freezing medium represents a turning point, as older studies reported that despite the protective effect of SL during freezing, a high percentage of SL is toxic to post-thaw viability and colony number.

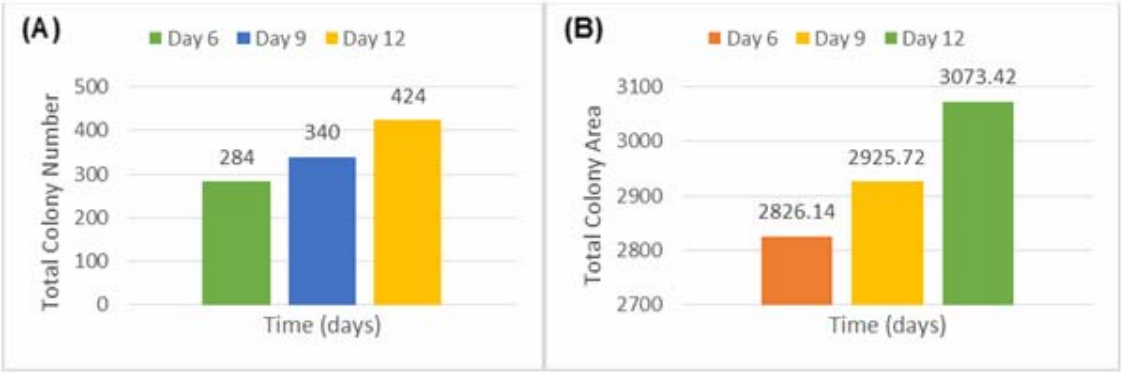

Figure 5 (A) Relationship between number of colonies and different culture days. Results from three independent experiments were used for each group. (B) Relationship between colony area (µm) and different culture days. Results from three independent experiments were used for each group

Thus, the toxic effect of this cryoprotectant is clearly taken into account in the current study and previous studies came to a similar conclusion (Forouzanfar *et al*. 2010; Salmani *et al*. 2014; Ustuner *et al*. 2014; Konyak *et al*. 2018).

The results observed showed that the number of colonies in the 0.5% SL treatment was significantly high compared to the other treatments. Due to the high cell viability in this group, the number of colonies will obviously also be high, and it also appears that SL increased colony formation by an unknown mechanism and time also affected colony proliferation, meaning that the number of colonies increased progressively after cultivation. In addition, researchers reported that Sertoli cells support germ cell colonization (Zargarzadeh *et al*. 2019). In the 1% SL group, the number of colonies during 12 days of co-cultivation showed no significant difference compared to the control group. The number of SSCs decreased in the 1.5% SL treated colonies as cell membrane damage and SL toxicity during freeze processing were the cause of the reduction in colony numbers (Ustuner *et al*. 2014; Mohebbi *et al*. 2021). In this way, SL and Sertoli cells promote germ cell proliferation through an unknown mechanism, so an increase in colony area was expected. During co-cultivation with Sertoli cells, the area of colonies in groups was larger by 0.5% than in other groups, with the colony area gradually increasing with each day of culture. The reason could be that the co-culture of spermatogonial cells with Sertoli cells shows a large increase in colony area. Time can also affect the size of the colonies. On the other hand, 1.5% SL has a negative effect on it. Furthermore, our results showed no significant difference in colony area size in 1% SL and control groups (Qasemi-Panahi *et al*. 2018; Zargarzadeh *et al*. 2019).

Finally, a number of important limitations need to be considered. First, the number of samples was relatively small, because the master's degree is short, but the cell culture and freezing take more time. Second, we did not have access to very advanced equipment in the laboratory to conduct the present study. Therefore, further research is needed to evaluate the lower percentages of SL (<0.5%) for cryopreservation of SSCs, and future investigations are needed to validate the kind of conclusions that can be drawn from this study.

CONCLUSION

To our knowledge, the current study is the first to evaluate the effects of SL on frozen and thawed Ghezel lamb SSCs. Our results indicate that the 0.5% SL group is an ideal extracellular cryoprotectant for the long-term preservation of SSCs as it results in a high percentage of cell viability, colony number and area. In addition, the Sertoli cell co-culture system can enhance *in vitro* colony formation of frozen and thawed spermatogonial cells. However, SSCs preserved in 1.5% SL have adverse effects due to their toxicity. Based on this, our current results demonstrate an improvement in cryostorage parameters of ram germ cells by supplementation with SL.

ACKNOWLEDGEMENT

The authors thank all laboratory colleagues who contributed to this article. We also want to thank the people from Tarbiat Modares and Tabriz University who supported this project. This work was supported by the Tarbiat Modares University, Tehran, Iran.

REFERENCES

Aliakbari F., Gilani M.A.S., Amidi F., Baazm M., Korouji M., Izadyar F., Yazdekhasti H. and Abbasi M. (2016). Improving the efficacy of cryopreservation of spermatogonia stem cells by antioxidant supplements. *Cell. Reprogram*. **18(2),** 87-95.

Aliakbari F., Sedighi Gilani M.A., Yazdekhasti H., Koruji M., Asgari H.R., Baazm M., Izadyar F., Kharrazi Nejad E., Khanezad M. and Abbasi M. (2017). Effects of antioxidants, catalase and α-tocopherol on cell viability and oxidative stress variables in frozen-thawed mice spermatogonial stem cells. *Artif. Cells. Nanomed. Biotechnol.* **45(1),** 63-68.

Chelucci S., Pasciu V., Succu S., Addis D., Leoni G.G., Manca M.E., Naitana S. and Berlinguer F. (2015). Soybean lecithin–based extender preserves spermatozoa membrane integrity and fertilizing potential during goat semen cryopreservation. *Theriogenology*. **83(6),** 1064-1074.

Dalmazzo A., Losano J.D.A., Rocha C.C., Tsunoda R.H., Angrimani D.R., Mendes C.M., Assumpção M.E.O.D.Á., Nichi M. and Barnabe V.H. (2018). Effects of soy lecithin extender on dog sperm cryopreservation. *Anim. Biotechnol.* **29(3),** 174-182.

El-Shahat K. and Hammam A. (2018). Effect of different types of cryoprotectants on developmental capacity of vitrified-thawed immature buffalo oocytes. *Anim. Reprod.* **11(4),** 543-548.

Fathi M., Zaher R., Ragab D., Gamal I., Mohamed A., Abu-El Naga E. and Badr M. (2019). Soybean lecithin–based extender improves Damascus goat sperm cryopreservation and fertilizing potential following artificial insemination. *Asian. Pac. J. Reprod.* **8(4),** 174-184.

Feng T.Y., Li Q., Ren F., Xi H.M., Lv D.L., Li Y. and Hu J.H. (2020). Melatonin protects goat spermatogonial stem cells against oxidative damage during cryopreservation by improving antioxidant capacity and inhibiting mitochondrial apoptosis pathway. *Oxid. Med. Cell. Longev.* **5,** 1-10.

Forouzanfar M., Sharafi M., Hosseini S., Ostadhosseini S., Hajian M., Hosseini L., Abedi P., Nili N., Rahmani H. and Nasr-Esfahani M. (2010). *In vitro* comparison of egg yolk–based and soybean lecithin–based extenders for cryopreservation of ram semen. *Theriogenology*. **73(4),** 480-487.

Ibtisham F. and Honaramooz A. (2020). Spermatogonial stem cells for *in vitro* spermatogenesis and *in vivo* restoration of fertility. *Cells.* **9(3),** 745-753.

Ibtisham F., Awang-Junaidi A.H. and Honaramooz A. (2020). The study and manipulation of spermatogonial stem cells using animal models. *Cell. Tissue. Res.* **380(2),** 393-414.

Ibtisham F., Zhao Y., Nawab A., Wu J., Mei X., Honaramooz A. and An L. (2021). *In vitro* production of haploid germ cells from murine spermatogonial stem cells using a two-dimensional cell culture system. *Theriogenology*. **162,** 84-94.

Izadyar F., Matthijs-Rijsenbilt J., Den Ouden K., Creemers L.B., Woelders H. and de Rooij D.G. (2002). Development of a cryopreservation protocol for type A spermatogonia. *J. Androl.* **23(4),** 537-545.

Jeong D., McLean D.J. and Griswold M.D. (2003). Long□term culture and transplantation of murine testicular germ cells. *J. Androl.* **24(5),** 661-669.

Kazemzadeh S., Mohammadpour S., Madadi S., Babakhani A., Shabani M. and Khanehzad M. (2022). Melatonin in cryopreservation media improves transplantation efficiency of frozen–thawed spermatogonial stem cells into testes of azoospermic mice. *Stem Cell Res. Ther.* **13(1),** 346-356.

Kim K.J., Lee Y.A., Kim B.J., Kim Y.H., Kim B.G., Kang H.G., Jung S.E., Choi S.H., Schmidt J.A. and Ryu B.Y. (2015). Cryopreservation of putative pre-pubertal bovine spermatogonial stem cells by slow freezing. *Cryobiology.* **70(2),** 175-183.

Konyak P., Mandal A., Mondal M., Bhakat C., Das S., Rai S., Ghosh M. and Karunakaran M. (2018). Preservation of black Bengal buck semen in soybean lecithin based chemically defined extender. *Indian J. Anim. Res.* **52(8),** 1151-1154.

Kubota H., Avarbock M.R. and Brinster R.L. (2004). Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. *Proc. Natl. Acad. Sci.* **101(47),** 16489-16494.

Mehdipour M., Kia H.D., Moghaddam G. and Hamishehkar H. (2018). Effect of egg yolk plasma and soybean lecithin on rooster frozen-thawed sperm quality and fertility. *Theriogenology*. **116,** 89-94.

Miguel-Jimenez S., Del Alamo M.M.R., Álvarez-Rodríguez M., Hidalgo C.O., Peña A.I., Muiño R., Rodríguez-Gil J.E. and Mogas T. (2020). *In vitro* assessment of egg yolk-, soya bean lecithin-and liposome-based extenders for cryopreservation of dairy bull semen. *Anim. Reprod. Sci.* **215,** 1-11.

Mirzapour T., Movahedin M., Tengku Ibrahim T.A., Haron A.W. and Nowroozi M.R. (2013). Evaluation of the effects of cryopreservation on viability, proliferation and colony formation of human spermatogonial stem cells *in vitro* culture. *Andrologia*. **45(1),** 26-34.

Mohebbi M., Moghaddam G., Qasemi-Panahi B., Daghigh Kia H., Rafat S. and Hamidian G. (2021). Cryopreservation of Spermatogonial Stem Cells of Native Goat of Iran. *Iranian J. Appl. Anim. Sci.* **11(2),** 351-356.

Motta J.P.R., Paraguassú-Braga F.H., Bouzas L.F. and Porto L.C. (2014). Evaluation of intracellular and extracellular trehalose as a cryoprotectant of stem cells obtained from umbilical cord blood. *Cryobiology.* **68(3),** 343-348.

Mousavi S.M., Towhidi A., Zhandi M., Amoabediny G., Mohammadi-Sangcheshmeh A., Sharafi M. and Hussaini S.M.H. (2019). Comparison of two different antioxidants in a nano lecithin-based extender for bull sperm cryopreservation. *Anim. Reprod. Sci.* **209,** 106171-106181.

Pirnia A., Assadollahi V., Alasvand M., Moghadam A. and Gholami M.R. (2020). A comparison of the effects of fetal bovine serum and newborn calf serum on cell growth and maintenance of cryopreserved mouse spermatogonial stem cells. *Mol. Biol. Rep.* **47(12),** 9609-9614.

Qasemi-Panahi B., Movahedin M., Moghaddam G., Tajik P., Koruji M., Ashrafi-Helan J. and Rafat S.A. (2018). Isolation and proliferation of spermatogonial cells from ghezel sheep. *Avicenna J. Med. Biotechnol.* **10(2),** 93-97.

Salmani H., Towhidi A., Zhandi M., Bahreini M. and Sharafi M. (2014). *In vitro* assessment of soybean lecithin and egg yolk based diluents for cryopreservation of goat semen. *Cryobiology.* **68(2),** 276-280.

SAS Institute. (2013). SAS®/STAT Software, Release 9.4. SAS Institute, Inc., Cary, NC. USA.

Shamsollahi M., Daghigh Kia H., Moghaddam G. and Taghizade A. (2018). Effects of centrifugation and different levels of soybean lecithin-based extender on post-thaw sperm quality of Ghezel ram semen. *Iranian J. Appl. Anim. Sci.* **8(2),** 257-262.

Sharafi M., Zhandi M. and Akbari Sharif A. (2015). Supplementation of soybean lecithin-based semen extender by antioxidants: Complementary flowcytometric study on post-thawed ram spermatozoa. *Cell. Tissue Bank.* **16(2),** 261-269.

Shi Y.Q., Wang Q.Z., Liao S.Y., Zhang Y., Liu Y.X. and Han C.S. (2006). *In vitro* propagation of spermatogonial stem cells from KM mice. *Front. Biosci.* **11(3),** 2614-2622.

Strober W. (1997). Trypan blue exclusion test of cell viability. *Curr. Protoc. Immunol*. **21(1),** 1-9.

Sun L., He M., Wu C., Zhang S., Dai J. and Zhang D. (2021). Beneficial influence of soybean lecithin nanoparticles on rooster frozen–thawed semen quality and fertility. *Animals.* **11(6),** 1769-1779.

Ustuner B., Alçay S., Nur Z., Sagirkaya H. and Soylu M.K. (2014). Effect of egg yolk and soybean lecithin on tris-based extender in post-thaw ram semen quality and *in vitro* fertility. *Kafkas Univ. Vet. Fak. Derg.* **20(3),** 393-398.

Wang P., Li Y., Hu X.C., Cai X.L., Hou L.P., Wang Y.F., Hu J.H., Li Q.W., Suo L.J., Fan Z.G. and Zhang B. (2014). Cryoprotective effects of low-density lipoproteins, trehalose and soybean lecithin on murine spermatogonial stem cells. *Zygote.* **22(2),** 158-163.

Zargarzadeh M., Tajik P., Movahedin M., Akbari G. and Qasemi-Panahi B. (2019). Immediate cryopreservation of isolated Ghezel ram spermatogonial stem cells and subsequent co-culture with Sertoli cells preserve their survival and functional capacity. *Eurasian J. Biosci.* **13(2),** 2614-2622.

Zhao X., Wan W., Zhang X., Wu Z. and Yang H. (2021). Spermatogonial stem cell transplantation in large animals. *Animals.* **11(4),** 257-262.