The association of trehalose and DMSO in cryopreservation of mouse cauda epididymal spermatozoa

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Abstracts

It has been shown that mouse sperm is more challenging to cryopreserve than the sperm of other mammals. The association of nonpermeating agents with penetrating agents reduces the concentration of penetrating agents, reducing their toxicity. This study evaluated the effect of the association of different concentrations of trehalose (0, 0.05 M, 0.1 M and 0.15 M) with 2.5% DMSO in the presence of 5 mg/mL human serum albumin (HSA). Accordingly, mouse spermatozoa samples collected directly into each CPA group were frozen/thawed under identical conditions. The assessments including viability, motility and DNA fragmentation rate were performed after one day, three days and seven days of freezing. Moreover, the fertilization function of spermatozoa in the group that maintained the best characteristics after cryopreservation was evaluated.

The results showed that a higher percentage of the parameters including viability, motility and DNA integrity rates were observed in the 0.1 M trehalose group. The in vitro fertilization results demonstrated that mouse spermatozoa frozen with the 0.1 M trehalose group were less fertile than those with fresh spermatozoa ($p < 0.05$). According to our research, the ideal parameters for cryopreservation were obtained when the cryopreserve medium contained 0.1 M trehalose in combination with DMSO.

Keywords: Cryopreservation, cryoprotectant agents, dimethyl sulfoxide, spermatozoa, trehalose.

Introduction

Almost all biomedical research uses mice and rats as animal models[3]. Despite being very simple to house and raise, transgenic or genetically modified strains of mice and rats are expensive to maintain and transport[12]. Moreover, spontaneous genetic mutations can also result from breeding[2]. Therefore, cryopreservation of sperm is one method for facilitating long-term storage and transit and lowering the expense of maintaining rodent strains[8]. Observations on the effects of freezing temperatures on human spermatozoa were possibly originally reported by Spallanzani in 1776[25]. With Polge's discovery of glycerol's cryoprotectant capabilities, more scientific advancement was accomplished considerably later[17].

According to Curry[4], the earliest offspring produced from cryopreserved spermatozoa was recorded in 1951 for cows, in 1953 for humans, in 1957 for pigs and horses and in 1967 for sheep[4]. Sperm cryobanks for cattle and humans were established in the 1960s and 1970s[19]. Cryopreserved spermatozoa are commonly used in human-assisted reproductive technologies and artificial insemination of animals nowadays[9]. Nevertheless, despite several successes in sperm cryobiology, efforts to find the best ways to recover viable spermatozoa following cryopreservation are still ongoing.

Preventing related cryodamage to sperm biological structures and functions, including the plasma membrane, mitochondria and chromatin, is one of the major challenges of cryopreservation[21]. Using cryoprotectant agents (CPAs) that protect sperm against injury in freezing media is a popular and effective strategy for preventing cryodamage. The cryoprotectant agents (CPAs) are typically separated into groups of nonpermeating as monosaccharide and disaccharide sugars (glucose, fructose, trehalose,…), polyvinylpyrrolidone and hydroxyethyl starch and permeating including glycerol, dimethyl sulfoxide (DMSO), ethylene glycol, methanol and amides based on their molecular weight, which is unable to cross or cross the plasma membrane respectively[18].

It was predicted that using both permeating and nonpermeating CPAs together would successfully cryopreserved spermatozoa. This hypothesis was confirmed by Fu et al[5] when they combined DMSO and trehalose in goose sperm preservation[5]. However, combining two types of CPA was ineffective for equine sperm cryopreservation[23]. Thus, the study of combining two types of CPAs to identify sperm cryopreservation's effectiveness again is needed.

On the other hand, the toxicity of permeating CPAs is a concern in cryopreservation. According to Galvao et al[6], there is a significant difference in toxicity depending on the concentration of DMSO, which is permeating CPA. It was discovered that DMSO decreased sperm viability via lowering ATP concentration as well as mitochondrial respiration, which resulted in cell death[6]. Reducing the

toxicity of permeating CPAs, such as DMSO, by reducing their concentration in combination with nonpermeating CPAs also needs to be evaluated in sperm cryopreservation.

This study aims to demonstrate the effect of trehalose in association with one permeating cryoprotectant, dimethyl sulfoxide (DMSO), on mouse cryopreserved spermatozoa.

Material and Methods

Experimental animals: A total of eighteen male mice (8-week-old) and six female mice (12-week-old) (*Mus musculus* Var. *Albino*) were used for spermatozoa and oocyte collection respectively. The animal experiment was approved by the Animal Care and Use Committee, University of Science, VNU-HCM, Vietnam (Approval No. 580B/KHTN-ACUCUS).

Experimental design: Two experiments were conducted including the comparison of the effectiveness of different concentrations of trehalose when combined with DMSO on mouse spermatozoa parameters in cryopreservation and the identification of the ability of *in vitro* fertilization of spermatozoa after thawing in the highest effect group. There were four freezing medium groups including 2.5% DMSO (Sigma-Aldrich, USA) combined with 0, 0.05 M, 0.1 M and 0.15 M of trehalose (Sigma-Aldrich, USA) in essential medium (DMEM-F12, Sigma-Aldrich, USA) and 5mg/mL human serum albumin (HAS, LifeGlobal, USA). Fresh spermatozoa were used as a control. The spermatozoa parameter evaluations including viability, motility and DNA fragmentation rate, were performed after one day, three days and seven days of freezing. The *in vitro* fertilization ability of spermatozoa was determined after seven days of cryopreservation.

Spermatozoa cryopreservation: Following euthanasia, both cauda epididymides were collected. Blood and fat were cleaned with sterile blotting paper (Pulppy, VN). The cauda epididymides were washed in PBS 1X (Gibco, USA) and then placed into 1.0 mL of freezing medium in a 4-well dish (Nunc, USA). Several small incisions allowed sperm to elute into the pre-warmed freezing medium for 10 minutes at 37°C[8, 22]. After incubation, the spermatozoa suspension (semen) was mixed and separated into two parts. One part was analyzed for spermatozoa parameters (viability, motility and DNA integrity rate) and the remaining part was cryopreserved in 1.8 mL cryotubes (Nunc, USA) with 50 µL per tube and ten samples per male mouse.

The filled cryotubes were kept at 4°C for 10 minutes for equilibration. Afterwards, the cryotubes were placed on liquid nitrogen vapour for 3 minutes, followed by being immediately plunged into liquid nitrogen (-196°C) and kept in storage for up to 7 days. Semen thawing was performed immediately before the sperm quality assessment. Briefly, cryotubes were thawed in a bath at 37°C until totally defrosted and then 200 µL of thawing medium (essential medium) was slowly added to these tubes and suspended gently. These tubes were incubated for 30 minutes at 37°C and 5% CO_2.

Spermatozoa viability: To assess spermatozoa viability, 10 µL of semen was mixed with 10 µL of live and dead dyes (a solution of 16 µM Calcein-AM and 8 µM Ethidium homodimer) (Sigma-Aldrich, USA). The suspension was incubated for 15 minutes at 37°C under 5% CO_2, protected from light. After that, stained semen was fixed on a slide (Marienfeld, Germany) and observed under the fluorescence microscope (Olympus, Japan). The percentage of live sperm in the visual field was recorded and calculated:

$$Spermatozoa\ viability = \frac{Live\ (giving\ off\ a\ green\ fluorescence)\ spermatozoa\ count}{Total\ spermatozoa\ count\ in\ visual\ field} \times 100\%$$

Spermatozoa motility: The spermatozoa motility was identified by taking 10 µL semen and placing it on a slide. The number of sperm moving in a straight line was observed using a light microscope (Zeiss, Germany). The percentage of sperm advancing was recorded:

$$Spermatozoa\ motility\ rate = \frac{Number\ of\ sperm\ advancing\ in\ a\ straight\ line}{Total\ spermatozoa\ count\ in\ visual\ field} \times 100\%$$

DNA fragmentation rate: The TUNEL assay evaluated spermatozoa nuclear DNA fragmentation rate according to the manufacturer's instructions. Briefly, spermatozoa were fixed with 200 µL of 4% (*w/v*) paraformaldehyde (Sigma-Aldrich, USA) (PBS, pH 7.4) for 15 minutes on ice after centrifugation (Hettich, Germany) at 3000 rpm for 10 minutes. The pellet of the fixed spermatozoa was collected. The spermatozoa were washed in cold PBS and fixed in 200 µL of 70% ethanol for 30 minutes at -20°C. Then, ethanol was removed and the samples were washed with 200 µL of wash buffer.

After centrifugation, spermatozoa were incubated in 50 µL of a DNA labelling solution for 60 min at 37ºC. The washing was done with 200 µL of rinse buffer and spermatozoa were incubated in 100 µL of antibody solution for 30 minutes at room temperature. Following this, 100 µL of 7-AAD / RNase A solution was added and incubated at the same conditions. Finally, the end solution was placed on a slide and observed by the fluorescence microscope:

$$DNA\ fragmentation\ rate = \frac{The\ number\ of\ spermatozoa\ that\ emit\ red\ fluorescence}{Total\ spermatozoa\ count\ in\ visual\ field} \times 100\%$$

***In vitro* fertilization:** The volume of 10 µL of thawed spermatozoa (5 – 12.5×10^6 cells/mL) was filled into a 40 µL drop of IVF medium (LifeGlobal, USA), which was

incubated overnight and then incubated for 30 minutes before IVF. Cumulus oocyte complexes (COCs) were collected from superovulated females by transferring them directly into this drop using a sterile needle (27 gauge). After 4 – 6 hours of co-culture, oocytes were washed and cultured in an embryo culture medium (LifeGlobal, USA) at 37°C and 5% CO_2 for 24 hours. The two-cell embryos were recorded by an inverted microscope (Olympus, Japan). Fresh spermatozoa were used as a control.

$$IVF\ rate = \frac{Number\ of\ two-cell\ embryos}{Total\ number\ of\ oocytes\ used} \times 100\%$$

Statistical analysis: The statistical analysis was performed with SPSS statistical software version 20 (IBM, USA). The graphs were shown using the GraphPad Prism 9.5.0 software (GraphPad, USA). The results were considered significant at the 5% level ($p < 0.05$) and expressed as mean ± SD.

Results

Spermatozoa viability: The thawed spermatozoa showed green and red fluorescence in all groups (Fig. 1). Therefore, spermatozoa were viable after cryopreservation. However, viability rates (%) of mouse spermatozoa were different with different concentrations of trehalose as shown in fig. 2. The results showed that the highest spermatozoa viability rate was in the 0.1 M trehalose and 2.5% DMSO group, which was 44.72 ± 1.25% ($p < 0.05$) after one day of cryopreservation.

However, it was still lower than in the control group (fresh semen) at 59.32 ± 3.76%. Significant differences were found between the 0.05 M trehalose and 2.5% DMSO group (34.98 ± 0.55%) and the 0.15 M trehalose and 2.5% DMSO group (36.88 ± 0.44%).

The added-trehalose groups all share the characteristic that sperm viability remained constant for up to 7 days ($p > 0.05$). Conversely, the lowest level of sperm viability was seen in spermatozoa cultured in a trehalose-free medium (30.13 ± 2.44%) ($p < 0.05$). Moreover, only in this group the viability rate decreased after the first three days ($p < 0.05$) and remained the same next time.

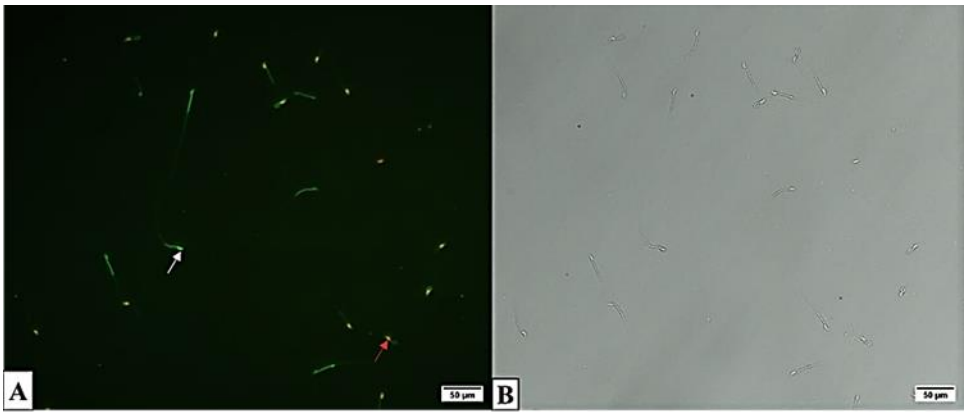

Figure 1: Spermatozoa live/dead staining after cryopreservation (×200)
(A) Visual field under fluorescence excitation light, (B)Visual field under white light
White arrow: live spermatozoa, red arrow: dead spermatozoa

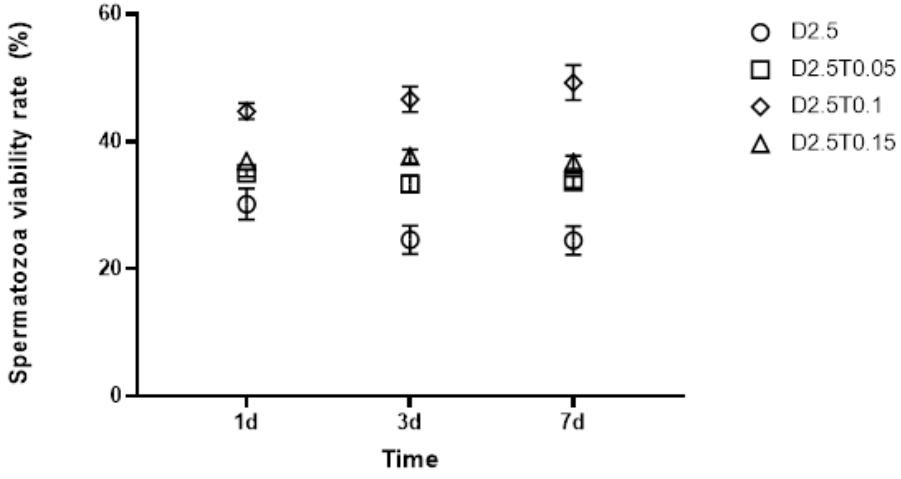

Figure 2: Spermatozoa viability rate after cryopreservation
D2.5: 2.5% DMSO group, D2.5T0.05: 2.5% DMSO and 0.05 M Trehalose group, D2.5T0.1: 2.5% DMSO and 0.1 M Trehalose group, D2.5T0.15: 2.5% DMSO and 0.15 M Trehalose group

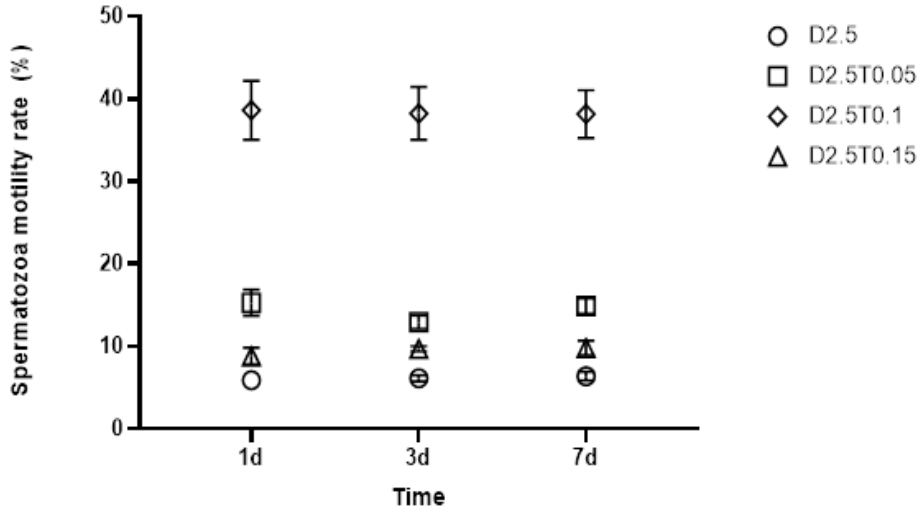

Figure 3: Spermatozoa motility rate after cryopreservation
D2.5: 2.5% DMSO group, D2.5T0.05: 2.5% DMSO and 0.05 M Trehalose group, D2.5T0.1: 2.5% DMSO and 0.1 M Trehalose group, D2.5T0.15: 2.5% DMSO and 0.15 M Trehalose group

Spermatozoa motility: The rate of spermatozoa motility was measured to determine the influence of cryopreservation. Post-preserved sperm in all groups had reduced motility compared to fresh sperm (56.84 ± 2.57%) ($p < 0.05$). The trehalose-supplemented groups had increased post-thaw motility in mouse spermatozoa at all survey times compared to the non-trehalose group. The maximum result for motility obtained with 0.1 M trehalose-containing media was 38.61 ± 3.56% ($p < 0.05$) after one day of cryopreservation, which is non-differentiation with those at the following times including 38.22 ± 3.19% and 38.14 ± 2.89% at days 3 and 7 respectively, as shown in fig. 3. This trend was similar in all groups.

Additionally, spermatozoa motility in the medium with a 0.05 M trehalose concentration was better than that with a concentration of 0.15 M trehalose during cryopreservation at 15.25 ± 1.59% and 8.80 ± 1.01% respectively. In summary, the results showed that cryopreservation time negatively affected spermatozoa motility in any of the groups ($p > 0.05$) and maximum motility was obtained using 0.1 M trehalose.

DNA fragmentation rate: Spermatozoa were stained with tunnel dye (emitting red fluorescence) after preservation in all groups as shown in fig. 4. However, there were differences between the groups in the frequency of red fluorescent sperm. The trehalose-free group (2.5% DMSO group) had the highest nuclear DNA fragmentation rate of spermatozoa at the time of the initial survey, reaching 21.92 ± 0.72% ($p < 0.05$).

This rate was similar in the two groups: 7.59 ± 0.21% and 7.59 ± 0.07% for the trehalose-supplemented group at 0.05 and 0.15 M concentrations respectively. The least amount of DNA fragmentation was found in spermatozoa preserved in a medium supplemented with 0.1 M trehalose at 4.37 ± 0.24% ($p < 0.05$). These rates were significantly increased ($p < 0.05$) compared with those of fresh semen (no fragmentation was recorded). This evaluation also found that sperm fragmentation was unaffected by cryopreservation time (Fig. 5).

***In vitro* fertilization:** The results of spermatozoa parameter evaluations including viability, motility and DNA integrity, showed that spermatozoa cryopreserved in the medium of 2.5% DMSO combined with 0.1 M of trehalose recovered the highest parameters. Therefore, thawed spermatozoa were collected after cryopreservation in this medium to determine their *in vitro* fertilization ability. The finding indicated that two-cell embryos were observed in both groups including thawed and fresh spermatozoa (Fig. 6). In addition, the study's fresh spermatozoa produced an *in vitro* fertility rate of 44.72 ± 0.40% higher than those in thawed spermatozoa (27.92 ± 0.92%).

Discussion

A valuable technique for protecting genetic resources is cryopreservation. This technique is necessary to maintain transgenic or genetically modified strains in animal models such as mice, commonly used in biomedical research. Even though cryopreservation is frequently employed to preserve fertility, one should not neglect its adverse effects such as motility issues, a decline in viability rate and other sperm parameter defects[7]. Using supplements with antioxidant activity such as sugars, during cryopreservation positively impacted sperm parameters. It is widely acknowledged that sugars affect the stability of biomolecules such as proteins and lipid membranes, particularly the disaccharide trehalose, a non-permeable cryoprotectant[10]. However, using only trehalose did not have an excellent sperm preservation effectiveness[1]. The previous study confirmed that trehalose, in association with DMSO, can be an ideal cryoprotectant solution and could decrease DMSO concentration in the typical DMSO medium[14]. Moreover, the combination of trehalose with permeable cryoprotectants such as glycerol and DMSO has shown positive outcomes in recent years[5].

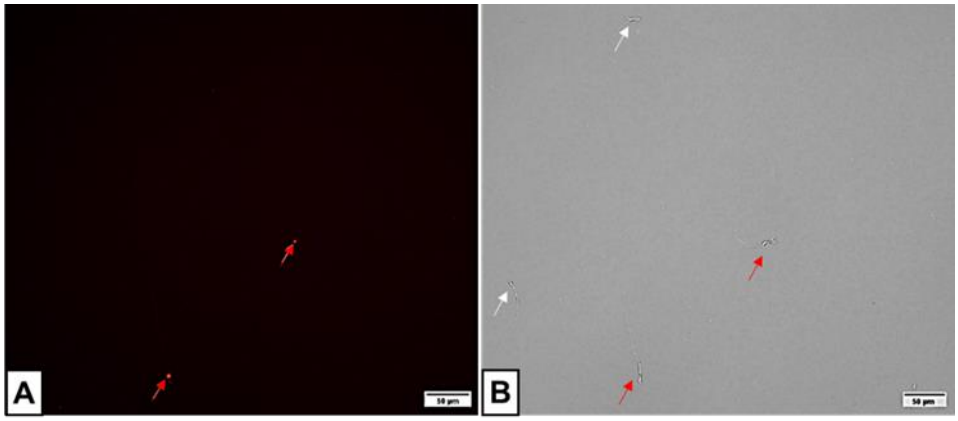

Figure 4: Spermatozoa Tunel staining after cryopreservation (×200)
(A) Visual field under fluorescence excitation light, (B) Visual field under white light
White arrow: normal spermatozoa, red arrow: spermatozoa with fragmented DNA

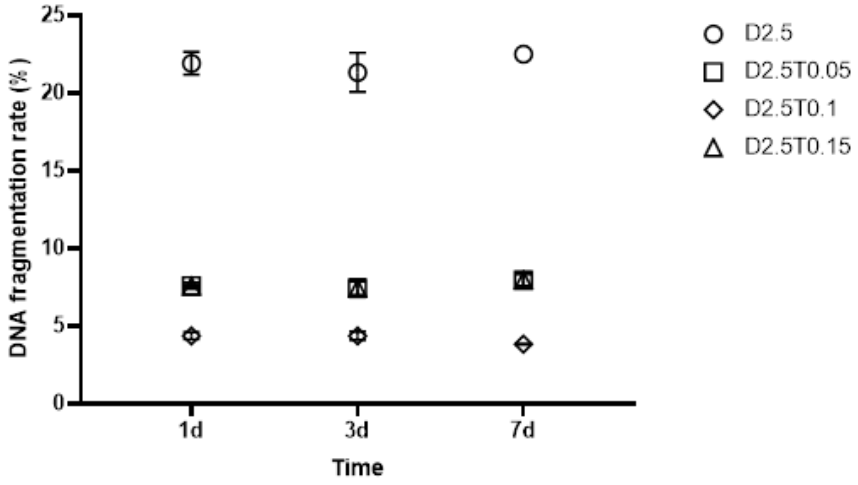

Figure 5: DNA fragmentation rate after cryopreservation
D2.5: 2.5% DMSO group, D2.5T0.05: 2.5% DMSO and 0.05 M Trehalose group, D2.5T0.1: 2.5% DMSO and 0.1 M Trehalose group, D2.5T0.15: 2.5% DMSO and 0.15 M Trehalose group

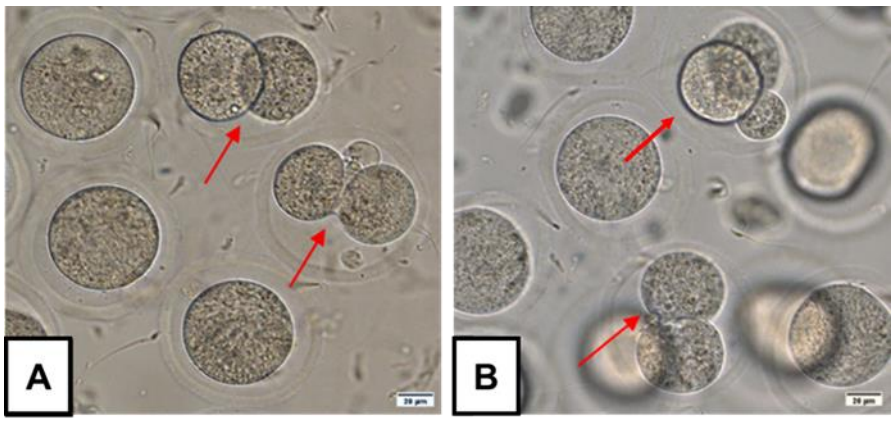

Figure 6: Two-cell embryos after in vitro fertilization
(A) Thawed spermatozoa group, (B) Fresh spermatozoa group

As suggested in several previous studies, DMSO has substantial toxicity. In addition, the high toxicity of DMSO in mouse sperm cryopreservation was observed in the publication of Park et al[16]. Additionally, it has been suggested to use DMSO alone at a low concentration (5%) to enhance the kinetics of engraftment, which can be further reduced by combining DMSO with another cryoprotectant solution[6]. In actuality, a low concentration of DMSO (2%) used in combination with sugar demonstrated a cryoprotective effect[14].

Therefore, in the present study, compared to the control group (trehalose-free group), adding different concentrations of trehalose to the 2.5% DMSO medium increased the vitality of mouse spermatozoa following thawing. This increase is consistent with previous studies by Zhu et al[26], Anjos et al[1], Izanloo et al[11] and Fu et al[5] in rabbit, Rassostrea angulata, turkey and goose semen, respectively[1,5,11,26]. Evidence indicated that DMSO increases membrane permeability at low doses (5%). In addition, the formation of water pores in biological membranes is induced at a concentration of 10% DMSO which is a commonly used dose in cryopreservation. It can be advantageous as cryoprotectants that encourage vitrification, can more easily replace intracellular water.

On the other hand, DMSO becomes more dangerous at increasing concentrations[24]. Selecting an appropriate cryoprotectant concentration is essential to maintain the integrity, structure and viability of cells after cryopreservation. This study showed that a low concentration of DMSO (2.5%) maintained spermatozoa parameters after cryopreservation when combined with trehalose. In addition, the results also demonstrated that the 0.1 M trehalose concentration was more effective at preserving sperm than the other two concentrations (0.05 M and 0.15 M trehalose). In the previous study, the addition of 100 mM trehalose improved post-thaw rabbit sperm parameters more than those of 0, 50, 75, 150 and 200 mM trehalose[26].

Moreover, trehalose at 0.05 M and 0.1 M ($p > 0.05$) in the concentration range of 0.025 M to 0.2 M is most effective concentration for the preservation of human sperm[7]. However, Zhu et al[26] found that the 0.05 M trehalose group protected the goose spermatozoa better than the 0.1 M trehalose group[5]. This difference is due to the initial cholesterol/phospholipid ratio of sperm from various species, which affects their optimal concentration range for cryoprotectants and is related to their species specificity[5]. The increase or decrease in osmolarity may be due to higher or lower concentrations of trehalose than 0.1 M respectively, leading to adverse effects on sperm parameters[13,20].

In this study, spermatozoa cryopreserved in the medium of 2.5% DMSO combined with 0.1 M of trehalose showed fertility after thawing. However, the effectiveness of fertilization was low (below 30%). The previous study showed that the sperm selection procedure before cryopreservation positively affects the cryo survival and fertilizing abilities of spermatozoa[15]. Improvements to the sperm inlet's quality should be made to consider the effect of subsequent cryopreservation.

Conclusion

Parameters of mouse spermatozoa were enhanced by adding several trehalose concentrations to the DMSO-containing cryomedia. Trehalose considerably improved spermatozoa viability, motility and DNA integrity rates in post-thawed spermatozoa compared to those stored in trehalose-free media. The current findings demonstrated the potential benefits of trehalose as a cryoprotectant agent which reduces DMSO toxicity when administered in low concentrations.

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