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Trehalose addition to a Tris-fructose egg yolk extender on quality of ram sperm preserved at 0 °C

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ABSTRACT - The present study investigated the effects of various concentrations of trehalose in Tris-fructose egg yolk diluent on ram semen preservation at 0 °C. Semen was collected by artificial vagina ejaculation from six rams of proven fertility. High-quality ejaculates were diluted with 0 (control), 5, 10, 15, and 20 mM trehalose of Tris-fructose egg yolk extender and control (tris-fructose egg yolk extender without trehalose), respectively. Then, the ejaculates were diluted to a concentration of $5{\times}10^8$ sperm/mL, cooled to 0 °C for 90 min, and maintained at that temperature for twelve days. The diluted semen samples were examined, and their sperm progressive motility, membrane functionality, and acrosome integrity recorded at 0, 24, 72, 144, 216, and 288 h. Two hundred ninety-six ewes were transcervically inseminated with the 216-h control (without trehalose) or the optimal trehalose concentration group semen, and the pregnancy and lambing rates were measured. No significant differences were established in the sperm progressive motility and membrane functionality among the control and 5, 10, 15, and 20 mM groups. The sperm samples of trehalose addition groups had no significant difference in the acrosome integrity of sperm, but they were, nonetheless, significantly higher than those in the control. No significant difference was detected in the lambing and pregnancy rates between the 5 mM and control groups. These results suggest that ram sperm is capable of fertilization after cooling and preservation at 0 °C by the use of 5 mM trehalose for Tris-fructose egg yolk diluent. Under these conditions, ram sperm can be more effectively preserved than under other four concentrations of diluents.

Keywords: diluent, ram, semen quality, trehalose

1. Introduction

In sheep industry, the preservation of semen is an important technique that ensures reproductive male ability in animal artificial insemination (AI). Ram sperm is susceptible to cold shock and is not easily stored frozen (White, 1993).

During cryopreservation, a large number of reactive oxygen species (ROS) are produced, which can destroy the structure and activity of the cell membrane and cause fatal sperm damage (Zhu et al., 2017). Unsaturated fatty acid content is among the constituents with the highest quantities of the sperm cell membrane, whereas there is a lack of antioxidants in the cytoplasm. During aerobic storage, free radicals such as superoxide anion, hydroxyl radical, and hydrogen peroxide affect the cell membrane of sperm under the action of lipid peroxidation (Sinha et al., 1996).

Trehalose (Tre) is a stabilizer of the lipid membrane, protein, and biofilm system. During the cryopreservation of sperm cells, it acts as a protective agent to reduce the formation of intracellular

ice crystals, to maintain protein structure, and significantly improve the mobility of sperm cells, as well as acrosome and cell membrane stability, reducing sperm cell aberrations or lesions (Wang and Dong, 2017). The addition of antioxidants to the cryoprotectant contributes to the protection of sperm cells from the adverse effects of reactive oxygen species and improves sperm cell recovery survival. In recent studies, trehalose has been added to the cryopreservation medium of various mammalian sperm cells, such as those of rabbits, rams, wild boars, goats, and bulls (Aisen et al., 2002; Aboagla and Terada, 2003; Tuncer et al., 2011; Gómez-Fernández et al., 2012; Zhu et al., 2017) to improve the survival rate of germ cells.

Although trehalose has been shown to play a significant role in semen preservation, the viscosity of the culture medium was found to increase with the elevation of trehalose concentration, which hampers sperm movement (Athurupana et al., 2015). Alternatively, the friction of sperm during swimming increased with the loss of free water in the cell. The latter condition inhibited the sliding of the microtubule filaments or other structural elements in the flagellum (Hu et al., 2009), and reduced motility. To the best of our knowledge, no exact additive concentration in sheep semen dilutions has been currently established. Our patented Tris diluent preserves sheep semen at 0 ℃ during long-term storage (Gao et al., 2008; Zhao et al., 2018; Wang et al., 2019).

The objectives of this study were to optimize the formulation of the diluent to further improve its preservation potential and investigate the effects of low doses (5, 10, 15, and 20 mM) of trehalose on sperm. This determines the optimal formulation of trehalose for long-term preservation of ram semen at 0 ℃.

2. Material and Methods

Animal research was conducted in compliance with the guidelines of the Animal Ethics Committee (SYXK 2018-009). The experiments were performed in Alar (40°54' N, 81°30' E), Xinjiang, China.

Semen samples from six mature Duolang rams (2-3 years of age) were used in this study. The physical condition and libido of the experimental animals were normal, and they were used for fertilization to obtain offspring by artificial insemination. In the course of the experiment, they were under the conditions of unified feeding and management. Rams were kept in a wide playground with free access to water. Besides the normal nutritional standards, they were supplementarily given carrots and eggs for 40 days before the experiment (Wang et al., 2014), and exercise was utilized to keep their normal physical condition. Six ejaculates were collected two times a week from each of the rams using artificial vagina ejaculation as previously described by Quinn et al. (1968).

Semen samples were immediately transferred into graduated test tubes after collection, placed in a thermo flask at 37 ℃, and transported to the laboratory for evaluation within 30 min. Ejaculates were included and evaluated in this study if the following criteria were met: volume of 0.5-2 mL, sperm concentration of $2×10^9$ sperm cells per mL; progressive motile sperm cell percentage higher than 80%; and abnormal sperm cell less than 10%. The semen samples were pooled to eliminate individual differences, divided into five equal aliquots, and kept at 37 ℃ in a water bath. After primary observation, the semen samples were diluted at a 1:4 ratio (semen: diluent, the concentration of $5×10^8$ sperm/mL) at 37 ℃ with different amounts of trehalose in each group: 0 (control), 5, 10, 15, and 20 mM. Within 90 min, the refrigerator (Midea, model: BCD-261WGM) was cooled down to 0 ℃, and ice cubes were added to the water bath for long-term storage of diluted semen until inspection.

The basic solution formula of the Tris diluent was as follows: double-distilled water (136 mL), egg yolk (40 mL), Tris (4.84 g), fructose (2.0 g), citric acid (2.68 g), adenosine triphosphate (ATP) (100 mg), bovine serum albumin (BSA) (300 mg), vitamin C (600 mg), penicillin (400,000 IU), and streptomycin (200,000 IU).

Sperm progressive motility was measured at 0, 24, 72, 144, 216, and 288 h after cooling. After viewing five different fields of the sample, sperm motility was estimated and noted by one observer. Then, we placed a drop (10 μL) of semen sample on a pre-warmed, grease-free slide. A cover slip

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was put over the drop, and it was subjected to examination using a high-power objective (40X magnification) of a microscope. One observer counted the total number of sperm cells moving in a straight line (≥200 sperm cells) in three visual fields at 37 ℃ (Zhu et al., 2015). Next, the progressive motility (Pm) of sperm cells was estimated by division of the number of sperm cells with a straight forward movement (Sfm) by the total number of sperm cells (Tns):

$$Pm\ (\%) = \frac{Sfm}{Tns} \times 100\% \tag{1}$$

Sperm acrosome integrity was measured at 0, 24, 72, 144, 216, and 288 h after cooling. The acrosome status was determined by adding a drop (10 μL) of the sample on a clean slide and making a smear. The sample was then fixed with 95% ethanol for 30 min and stained with hematoxylin for 20 min, followed by rinsing with distilled water. After dyeing with eosin staining solution for 5 min, the purified water was washed and naturally air-dried. Further, the stained sample was examined by a (400X) microscope. We counted the total number of sperm cells in different fields (≥200 sperm cells) and the number of intact sperm cells. Additionally, the sperm acrosome integrity rate (Ari) was estimated by the acrosome intact sperm cell count (Aic) divided by the sperm cell count (Sc):

$$Ari\ (\%) = \frac{Aic}{Sc} \times 100\% \tag{2}$$

Sperm cell membrane functionality was measured by hypoosmotic swelling test (HOST) at 0, 24, 72, 144, 216, and 288 h after cooling. Semen samples (10 μL) were mixed with HOST solution (100 μL) and incubated at 37 ℃ for 30 min. After incubation, 20 μL of the mixture was spread with a cover slip on a warm slide under light microscopy at 400X magnification. At least 200 spermatozoa per slide were observed. The sperm cells with swollen tails were considered as functional. Moreover, the sperm cell membrane functionality rate (Mfr) was estimated by number of sperm cell expansion (Nse) divided by the total sperm cell count (Tsc):

$$Mfr\ (\%) = \frac{Nse}{Tsc} \times 100\% \tag{3}$$

We re-collected and preserved semen for artificial insemination. Duolang ewes (n = 296) housed in a free pasture were used for transcervical insemination. The ewes were synchronized with a CIDR-based protocol for 14 days. Fifty-eight hours before the artificial insemination, the sponges were removed, and the animals were given an intramuscular injection of 500-600 IU of equine chorionic gonadotropin. Each ewe was inseminated twice with semen stored for 216 h.

Ewes were randomly assigned to an optimal trehalose concentration group (n = 156), consisting of ewes inseminated with the optimal trehalose concentration diluent and a control group (n = 140), composed of ewes inseminated with the control diluent. Semen from the aforementioned six rams was randomly assigned to ewes and equally distributed for AI. Detection of pregnancy was performed by B-mode ultrasonography on the 40th day. Pregnancy and lambing rates were recorded as an index of fertility and reproductive performance. Moreover, the pregnancy rate (Pr) was estimated by the number of gravid ewes (Nge) divided by the total number of inseminated ewes (Tni):

$$Pr\ (\%) = \frac{Nge}{Tni} \times 100\% \tag{4}$$

Finally, the lambing rate (Lr) was estimated by the number of lambing ewes (Nle) divided by the total number of inseminated ewes (Tni):

$$Lr\ (\%) = \frac{Nle}{Tni} \times 100\% \tag{5}$$

The sperm progressive motility, membrane functionality, and acrosome integrity rates were presented as mean ± standard error of mean (SEM). Data were analyzed using ANOVA, followed by LSD and Duncan tests for determination and comparison of the differences between the treatments, conducted by SPSS software (SPSS Inc., Chicago, IL, USA). The pregnancy and lambing rates were assessed via chi-square test. The significance level of 5% was considered in all statistical analyses.

3. Results

The sperm progressive motility did not change significantly with the increase in the trehalose dilution concentration in six different analyzed times. However, the sperm progressive motility of different trehalose concentrations showed a downward trend with time. The ram sperm progressive motilities of these were still higher than 50% at 216 h (Table 1).

In the six time periods (Table 2), the sperm acrosome integrity rate of control was significantly lower than that of the other groups (P<0.05); the acrosome integrity rate of each group showed a downward trend with the prolongation of time, but did not display significant changes with the increase of trehalose dilution.

The sperm cell membrane functionality rate did not change with the increase of trehalose concentration in the six time periods (Table 3). On the other hand, the membrane functionality rate of the sperm cells in the five groups showed a significant downward trend with time. Nevertheless, the membrane functionality rate of the ram sperm was still higher than 60% at 216 h.

The control group and the 5 mM group were preserved at 0 °C (Table 4). At 216 h, 140 ewes were artificially inseminated with control groups, and 92 were pregnant with a pregnancy rate of 65.7%. A total number of 156 ewes were inseminated with semen criopreserved by 5 mM trehalose, of which 103 were pregnant, with a pregnancy rate of 66.0%. Therefore, neither the addition of trehalose nor the

Table 1 - Progressive motility of Duolang ram sperm, stored at 0 °C in diluents supplemented with different levels of trehalose (mean±SEM)

Group	0 h	24 h	72 h	144 h	216 h	288 h
Control	87.7±2.0	83.7±2.8	78.1±2.2	70.8±4.2	61.1±3.6	37.6±2.8
Tre (5 mM)	86.8±1.9	83.0±1.7	77.5±2.5	71.8±2.3	56.6±2.3	36.2±3.3
Tre (10 mM)	86.0±2.8	82.5±2.1	76.1±1.2	70.2±2.6	56.6±2.1	35.8±1.6
Tre (15 mM)	85.4±1.6	81.9±1.6	74.0±1.5	65.6±3.7	55.2±3.5	37.0±4.1
Tre (20 mM)	85.9±1.7	81.3±0.9	75.8±3.1	71.7±3.1	55.0±3.9	34.8±2.4

Control - Tris-fructose egg yolk extender without trehalose; Tre - trehalose.

Table 2 - Acrosome integrity of Duolang ram sperm in diluents supplemented with different levels of trehalose (mean±SEM)

Group	0 h	24 h	72 h	144 h	216 h	288 h
Control	89.4±2.7a	81.5±1.1b	75.7±5.7b	65.4±2.8b	62.6±2.5b	51.3±1.8b
Tre (5 mM)	88.7±1.8a	86.0±1.3a	84.7±0.8a	80.6±0.7a	77.9±4.5a	73.9±5.4a
Tre (10 mM)	89.8±5.2a	86.3±3.4a	83.0±1.8a	82.7±1.9a	78.6±3.2a	74.3±2.5a
Tre (15 mM)	90.5±3.8a	85.7±1.1a	82.2±0.8a	81.7±0.5a	79.6±2.4a	75.8±5.2a
Tre (20 mM)	88.0±3.9a	86.0±1.3a	82.1±2.2a	81.9±2.3a	79.7±4.4a	75.2±4.9a

Control - Tris-fructose egg yolk extender without trehalose; Tre - trehalose.
a,b - Different letters mean statistically different results (P<0.05).

Table 3 - Sperm membrane functionality of Duolang ram sperm in diluents supplemented with different levels of trehalose (mean±SEM)

Group	0 h	24 h	72 h	144 h	216 h	288 h
Control	93.9±1.4	87.6±5.1	83.3±3.9	74.7±3.9	60.1±5.9	33.6±1.7
Tre (5 mM)	92.1±3.4	88.6±4.3	80.6±4.5	68.6±3.7	61.2±2.4	38.5±1.7
Tre (10 mM)	90.7±5.8	87.1±4.4	77.8±3.2	66.7±4.2	62.9±3.0	35.3±2.4
Tre (15 mM)	89.0±4.9	85.9±4.2	76.9±3.5	69.2±6.3	62.0±3.1	34.2±1.9
Tre (20 mM)	87.9±5.2	87.4±4.0	79.3±5.4	67.5±2.3	56.7±2.8	34.4±4.3

Control - Tris-fructose egg yolk extender without trehalose; Tre - trehalose.

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Table 4 - Effect of the presence of trehalose in the extender on in vivo results after artificial insemination of semen preserved to 216 h

Group	Total number of inseminated ewes	Pregnancy rate (%)	Lambing rate (%)
Control	140	92 (65.7)	87 (62.1)
Tre (5 mM)	156	103 (66.0)	98 (62.8)

Control - Tris-fructose egg yolk extender without trehalose; Tre - trehalose.

absence of trehalose in the dilution had a significant effect on the sheep pregnancy rate of ($P>0.05$). No significant difference was detected in the sheep lambing rate between the control group (62.1%) and the 5-mM group (62.8%).

4. Discussion

In this study, we investigated the effect of trehalose on the characteristics of sperm in Duolang rams after low-temperature storage conditions. The results showed that the addition of trehalose at a concentration lower than 20 mM in the Tris-fructose egg yolk semen dilution did not directly increase the sperm progressive motility of the preserved semen, but significantly increased the integrity of the preserved sperm acrosome. Tris patented semen diluent effectively preserved ram semen for more than 216 h without trehalose, considering that artificial insemination can be successfully performed by sperm cell viability higher than 50%. The addition of low doses of trehalose effectively protected the sperm acrosome. Our observations indicate that the addition of trehalose to the Tris-patented diluent improves the quality of long-preserved, diluted, fresh ram semen under low-temperature conditions.

The detection of sperm acrosome integrity rate is routine in sperm cell morphology and functional examination. Trehalose supplements significantly improved Duolang ram semen acrosome integrity. In addition, the trehalose supplementation significantly enhanced the sperm progressive motility and the level of intact acrosome membranes as compared with those of the control group. The reasons for this difference are the various amounts of trehalose added and the different basic additives, or the different species and preservation methods. Similar results were obtained in pigs by Gutiérrez-Pérez et al. (2009). Therefore, low concentrations of trehalose effectively protect sperm. As previous studies have shown, the cryoprotective activity of antioxidant additives on sperm is within a certain range of concentration. Exceeding this concentration range of antioxidant additives was found to boost the permeability of sperm, which affected sperm motility, membrane integrity, and fertility (Bucak et al., 2007). Our results also confirmed that the acrosome integrity rate of the ram sperm cell membrane was considerably improved by the addition of trehalose. However, there was no significant difference between the four doses of the addition below 20 mM, suggesting that the optimized trehalose additive amount is 5 mM. Therefore, adding the proper amount of trehalose to the fresh ram semen can improve the sperm acrosome integrity rate. During the sperm treatment, trehalose binds to the sperm cell membrane, which reduces the damage of the sperm caused by dehydration and the damage to the acrosomal and plasma membranes, thus stabilizing sperm cell membrane structure (Nayernia et al., 2002; Zhu et al., 2017). The proteolytic enzymes (acrosome enzymes) existing in sperm acrosomes are critically involved in the fertilization process (He et al., 2016; Chuawongboon et al., 2017). The detection of the acrosome integrity can indirectly reflect the sperm function, which is of great significance in the evaluation of abnormal sperm function and artificial insemination effectiveness.

In our investigation, the sperm acrosome integrity rate of the trehalose-added dilution was significantly higher than that of the non-addition group, and the trehalose protective effect was more obvious with time. This was due to the non-specific protective effect of trehalose on biomolecules and the accumulation of trehalose in the cells of some organisms in an attempt to cope with extreme external drought and osmotic stress (Han et al., 2016). Sperm is exposed to reactive oxygen species during

cryopreservation, and the antioxidant action of trehalose has a preservation effect against their damage. Moreover, trehalose supplies energy to sperm during sperm storage (Watson et al., 1979), enabling long-term storage. Whether the same effect would occur at a lower concentration needs to be elucidated in further studies.

5. Conclusions

Based on the results of the present study, the supplementation with trehalose in the semen extender significantly improves sperm acrosome integrity. However, no effect is exerted on sperm progressive motility, membrane functionality, and pregnancy and lambing rates. Importantly, we determined that the addition of a dilution of 5 mM trehalose is optimal for the long-term storage of fresh ram semen at 0 °C.

Author Contributions

Conceptualization: Q. Gao. Data curation: G. Xiao, D. Fang and N. Li. Formal analysis: D. Fang and N. Li. Supervision: C. Han and Q. Gao. Writing-original draft: J. Zhao, W. Zhu and C. Han. Writing-review & editing: J. Zhao and Q. Gao.

Acknowledgments

Conflict of Interest

The authors declare no conflict of interest.

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