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Determination of the effects of trehalose and iodixanol on kinematic values in frozen ram sperm after thermal stress test

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ABSTRACT

Artificial insemination is the most practical and economical method for breeding in domestic animals. However, certain physiological characteristics of sheep and the adverse effects that occur after thawing frozen ram semen hinder its widespread use. In the presented study, the effects of extenders created using different combinations of iodixanol and trehalose, in addition to a Tris-based extender, on frozen ram semen were investigated. For this purpose, thermal stress tests were performed using frozen and thawed ram semen, and their effects on spermatozoa kinematic values were determined using computerized analysis devices. VAP, VCL, and ALH values were found to be similar in all groups. The group with the highest VSL value was OP5, and the difference between this group and the TR50/OP2.5 and TR50/OP5 groups was found to be significant. The group with the highest BCF values was the control group, and the differences between this group and the TR50/OP 1.25, TR50/OP 2.5, TR50/OP 5, and OP5/TR25 groups were found to be significant. The highest STR value was found in the TR25 group, and the differences between this group and the TR50/OP 2.5 and TR50/OP 5 groups were found to be significant. The highest LIN value was obtained from the TR50/OP 1.25 group, and the differences between this group and the TR50/OP 2.5 and TR50/OP 5 groups were found to be significant. In conclusion, changes in LIN, VSL, and STR values, which may affect sperm motility, indicate that iodixanol and trehalose, when used in appropriate proportions, have the potential to positively impact spermatological data. However, more comprehensive studies are needed to clearly determine these effects.

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Introduction

Artificial insemination is the most important tool used to increase breeding rates. Frozen semen produced from an adult ram in one week can inseminate 500-1000 ewes. Sperm freezing, when combined with artificial insemination, is a crucial biotechnological method used to preserve and disseminate high-quality genetic material in the field (Gökçen 1982). However, the inability to penetrate the cervix during insemination in sheep due to their anatomical structure and the failure to successfully freeze ram semen are the most significant obstacles to the widespread adoption of artificial insemination (Marco-Jiménez et al. 2005; Ak et al. 2010). One of the most important steps in a successful artificial insemination is the freezing of semen. However, during this stage, spermatozoa are negatively affected by the harmful effects of cold (cold shock), and pregnancy rates decrease (Ak et al. 2010). Unfortunately, ram semen is more sensitive to cold shock than other domestic animal species (Fiser et al. 1987; Bacınoğlu et al. 2007; Ak et al. 2010). One of the key factors in successful sperm freezing is the freezing method. However, in addition to the method used, the composition and ingredients of the sperm extender are crucial for the sperm cells' survival during freezing (Hammerstedt et al. 1990; Curry et al. 1994; Salamon and Maxwell 2000; Purdy 2006). Although various

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researchers have developed different extenders and freezing methods, the desired pregnancy rates have not yet been achieved under field conditions (El-Alamy and Foote 2001; Morrier et al. 2003; Nur et al. 2010). Therefore, it is emphasized that the development of new and better semen extenders is vital for increasing pregnancy rates in artificial insemination in sheep (Aisen et al. 2000). Despite extensive efforts in recent years, it is reported that very few new substances have been discovered for use in freezing ram semen (Yániz et al. 2005). Trehalose, one of these substances, is one of the most effective substances used in freezing ram semen (Aisen et al. 2002; Matsuoka et al. 2006; Berlinguer et al. 2007; Bucak et al. 2007). Cirit et al. (2013) tested iodixanol, an X-ray contrast agent, for the first time in the world for the freezing of ram semen and reported that it protected the semen better than trehalose against the adverse effects of freezing ($p<0.05$). Exposing semen to high temperatures after thawing increases the speed of all biological processes and spermatozoa (Herman and Madden 1972; Paul et al. 1985). Therefore, the Thermal Stress Test (TST), based on the principle of short-term incubation of semen at high temperatures, has been used in the past to determine spermatozoa durability. It has been suggested that it can determine the viability of cells in vivo by affecting motility (Fiser et al. 1991; Tardif et al. 1999; Taş et al. 2006; Bacinoglu et al. 2008). Among the average speed parameters of spermatozoa, VCL (Velocity Curve Linear) expresses the average speed of the spermatozoon along its path as µm/s, VSL (Velocity Straight Line) expresses the average speed from the beginning to the end of the path followed along a straight line as µm/s, and VAP (Velocity Average Path) expresses the average speed of the spermatozoon during the total path traveled as µm/s. In addition, STR (Straightness %) represents the distance traveled by spermatozoa in a straight line (VSL/VAP X 100), LIN (Linearity %) represents the linear motion of spermatozoa (VSL/VCL X 100), and WOB (Wobble %) represents the degree of wobbling of spermatozoa from side to side (VAP/VCL X 100) during the total distance traveled (Verstegen et al. 2002; Foote 2003; Malo et al. 2006).

Material and Methods

The presented study was approved by the local ethics committee of the Istanbul University Faculty of Veterinary Medicine (2014/38). The study used healthy 2-4 year old Kıvırcık rams, housed in closed enclosures under the same care and feeding conditions, as live subjects. Semen samples were collected from the rams in the department twice, 3 days apart, before the study, and their spermatological characteristics were examined. Five rams with the best spermatological characteristics were selected for use in the study. The animals were fed 1 kg of concentrate feed and high-quality hay per ram daily, and clean water was available at all times. Sperm collection studies were conducted between January and June (outside the breeding season).

Preparation of sperm extenders

All chemicals used in the preparation of extenders, except iodixanol, were supplied by Sigma Chemical Co. (Saint Louis, MO, USA) unless otherwise stated. OptiPrep™ (60% solution of iodixanol in water, w/v) was used as the iodixanol source (Axis-Shield PoC AS, Oslo, Norway). In the study, a 15% egg yolk solution in bidistilled water was prepared and then centrifuged for 45 minutes at 4100 rpm in a refrigerated centrifuge (Nüve, NF400) to remove coarse particles. Extenders were prepared using this solution. Tris-based extender [Tris 27.1 g/L, citric acid 14 g/L, fructose 10 g/L, egg yolk 15% (v/v)] was used as the primary extender in the study. The glycerol-free basic extender (285 mOsm, pH 6.8) was divided into nine equal volumes and, excluding the control group, different concentrations of iodixanol and trehalose were added to form the extender groups as follows.

Group 1 (Control group, Tris): Tris
Group 2 (OP5 group): Tris + 5.0% iodixanol
Group 3 (TR25 group): Tris + 25 mM trehalose
Group 4 (TR50 group): Tris + 50 mM trehalose
Group 5 (TR50/OP1.25 group): Tris + 50 mM trehalose + 1.25% iodixanol
Group 6 (TR50/OP2.5 group): Tris + 50 mM trehalose + 2.5% iodixanol
Group 7 (TR50/OP5 group): Tris + 50 mM trehalose + 5.0% iodixanol
Group 8 (TR25/OP5 group): Tris + 25 mM trehalose + 5.0% iodixanol
Group 9 (TR12.5/OP5 group): Tris + 12.5 mM trehalose + 5.0% iodixanol

After the extenders were prepared, they were filtered through 0.22 µm disposable filters (Millex gp, 0.22 µm) to prevent coarse particles from causing problems during analysis on the computerized semen analyzer and stored at +4°C until use. The prepared extenders were used for a maximum of two semen collections, and the extenders were reconstituted after every two semen collections. Each extender prepared as above was divided into two equal portions and labeled "parts A and B." 10% glycerol was added to the extenders labeled "parts B." Thus, the total glycerol content used in semen dilution was set at 5% (Cirit et al. 2013).

Semen collection

Semen was collected from the rams twice a week using an electroejaculator. The rams were placed in a suitable lateral position, and the preputial hairs were trimmed with scissors, and the preputial tip was

cleaned with saline solution. 2-10 volt electric voltage was applied for 5 seconds via a probe inserted into the rectum, followed by a 5-second rest period. The semen in the penile canal was manually directed out of the preputium and massaged out during the rest periods. The semen was collected in 50 ml plastic tubes and transported to the laboratory immediately. The semen samples were then individually analyzed for volume, mass movement, motility, and sperm concentration. Sperm concentration was determined using an automatic concentration device (IMV, Accucell). Mass motility was examined by placing fresh semen on a slide at 37°C, then using a heated phase-contrast microscope at 40x magnification without covering the coverslip. A scoring system of 0-4 was used (Ak et al. 2010). Only good-quality semen samples (volume: ≥0.5 mL; mass motility: ≥3; motility: ≥70%, sperm concentration: ≥2x10^9/ml) were used in the study. To eliminate individual differences, good-quality semen samples were mixed (pooled) and divided into nine equal portions, each group diluted with its own extender.

Diluting and freezing sperm

A two-stage dilution method was used for dilution of semen (Ak et al. 2010). Sperm samples were slowly diluted to a concentration of 400 million spermatozoa per milliliter with glycerol-free extenders (Extender A portion) held in a water bath set at 24°C (room temperature). The temperature of the diluted semen samples was lowered to +5°C by adding ice cubes to the water bath at a cooling rate of 0.25°C per minute (1°C every 4 minutes). After cooling, motility analyses were performed again, and the second dilution stage was initiated. Sperm samples were diluted with 10% glycerol-containing extenders (Extender B portion) previously cooled to +5°C in a cold display case set at +5°C. B extenders were added to each sample, equal to the total volume of the diluted semen in the first stage, thus diluting the semen to a total of 200 million spermatozoa per milliliter.

The second dilution stage was completed in three steps. First, 20%, then 30%, and finally the remaining 50% of the B extenders were slowly added to the samples at five-minute intervals to complete the second dilution stage (Cirit et al. 2013). The diluted semen samples were then stored in a cold display case set at +5°C for 1 hour for equilibration (Cirit et al. 2013). At the end of equilibration, the samples were drawn onto 0.25 ml clear plastic straws, and the ends were sealed with polyvinyl alcohol. The straws were arranged on steel straw stacking combs and frozen in nitrogen vapor at a height of 4 cm above the liquid nitrogen level for 10 minutes. The frozen straws were stored in liquid nitrogen until the day of the study. The semen collection and freezing procedures were repeated a total of 10 times during the study.

Thermal stress test

For the TST test, frozen semen was thawed and incubated in 2-ml capped plastic (Eppendorf) tubes in an oven set at 46°C for 15 minutes (Bacinoglu et al. 2008; Cirit et al. 2013). At the end of incubation, samples were collected immediately, and VAP, VCL, ALH, VSL, BCF, STR, and LIN values were recorded using a semen analyzer. Semen analysis was repeated three times for each sample.

Statistical analysis

Statistical analyses were performed using SPSS program version 11.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to determine the differences between the groups in the data obtained in the study, and Duncan's Multiple Range test was used to control the significance of the differences (Duncan 1955).

Results

The kinematic values obtained after thermal stress testing for the study groups after thawing are shown in Table 1. Analyzing the table reveals that while there were no differences in VAP, VCL, and ALH values, there were differences between the groups in VSL, BCF, STR, and LIN values.

Table 1. Comparison of kinematic properties of spermatozoa after post-thaw Thermal Stress Test (TST) between groups

Group Number	Group Name	VAP (µm/sn)	VSL (µm/sn)	VCL (µm/sn)	ALH (µm/sn)	BCF (Hz)	STR (%)	LIN (%)
1	Control	76.3 ± 3.51	63.4 ± 3.35^{ab}	135.0 ± 6.12	7.0 ± 0.40	34.5 ± 0.56^{d}	80.1 ± 2.10^{abc}	49.4 ± 2.64^{bc}
2	OP5	83.1 ± 1.85	69.1 ± 1.68^{b}	144.1 ± 4.30	6.8 ± 0.38	34.1 ± 0.71^{cd}	80.8 ± 1.13^{abc}	48.6 ± 1.39^{abc}
3	TR25	79.8 ± 2.58	67.1 ± 2.11^{ab}	138.3 ± 5.23	6.8 ± 0.34	33.6 ± 0.53^{bcd}	82.3 ± 0.62^{c}	50.2 ± 0.91^{c}
4	TR50	77.5 ± 2.20	64.4 ± 1.75^{ab}	133.8 ± 4.65	6.6 ± 0.35	32.9 ± 0.60^{abcd}	80.7 ± 1.01^{abc}	48.8 ± 1.03^{abc}
5	TR50/OP1.25	74.8 ± 2.39	62.3 ± 1.66^{ab}	127.6 ± 4.77	6.3 ± 0.34	32.4 ± 0.63abc	81.8 ± 0.75^{c}	50.7 ± 1.00^{c}
6	TR50/OP2.5	76.1 ± 3.43	61.3 ± 2.83^{a}	136.2 ± 6.17	6.8 ± 0.35	31.9 ± 0.55^{ab}	77.6 ± 0.57^{a}	44.9 ± 0.50^{a}
7	TR50/OP5	75.0 ± 3.08	60.0 ± 2.46^{a}	134.4 ± 6.09	6.6 ± 0.35	31.2 ± 0.92^{a}	78.1 ± 0.49^{ab}	45.5 ± 0.61^{ab}
8	OP5/TR25	76.2 ± 2.86	63.4 ± 2.04^{ab}	132.3 ± 6.22	6.6 ± 0.37	31.9 ± 0.69^{ab}	81.6 ± 0.89^{c}	49.3 ± 1.14^{bc}
9	OP5/TR12.5	78.6 ± 2.81	65.2 ± 2.07^{ab}	136.2 ± 6.10	6.7 ± 0.36	33.0 ± 0.65^{abcd}	81.2 ± 1.00^{bc}	50.0 ± 1.35^{c}
	P Values	p>0.05	p<0.05	p>0.05	p>0.05	p<0.05	p<0.05	p<0.05

Values are expressed as Mean ± Standard Error (Mean ± SE). abcd: Differences between values bearing different letters in the same column are statistically significant

Discussion and Conclusion

In semen exposed to high temperatures after thawing, the speed of all biological processes and spermatozoa increases (Herman and Madden 1972; Paul et al. 1985). Therefore, the thermal stress test (TST), based on the principle of short-term incubation of semen at high temperatures, has been used in the past to determine spermatozoa durability and has been suggested to determine the viability of cells in vivo by affecting motility (Fiser et al. 1991; Tardif et al. 1999; Taş et al. 2006; Bacinoglu et al. 2008). We could not find any other study demonstrating the change in kinematic values in ram sperm subjected to thermal stress testing as applied in this study. Cirit et al. (2013) reported that the addition of 50 mM trehalose to ram semen extender significantly increased total motility after the TST, but Özmen et al. (2020) reported no difference in total and progressive motility rates after the TST. Motility values were not examined in the presented study, but contrary to expectations, no difference was found between the 50 mm group and the other study groups in terms of kinematic values. This may be due to the different breeds of rams used in the studies. VAP, VCL, and ALH values were found to be similar in all groups. The group with the highest VSL value was OP5, and the difference between this group and the TR50/ OP2.5 and TR50/OP5 groups was found to be significant. The group with the highest BCF value was the control group, and the differences between this group and the TR50/OP1.25, TR50/OP2.5, TR50/OP5, and OP5/TR25 groups were found to be significant. The highest STR value was found in the TR25 group, and the differences between this group and the TR50/OP2.5 and TR50/OP5 groups were found to be significant. The highest LIN value was obtained from the TR50/OP1.25 group, and the difference between this group and the TR50/OP2.5 and TR50/OP5 groups was found to be significant. Increases in LIN, VSL, and STR values indicate that iodixanol and trehalose, when used in appropriate proportions, have the potential to positively impact spermatological data. Increases in LIN and VSL values may specifically affect sperm speed, time to reach the fertilization site, and motility. This will impact the fertilization potential of thawed ram sperm. In conclusion, the study suggests that the addition of trehalose and iodixanol to semen extenders may affect kinematic values after thermal stress testing, but further studies would be beneficial to definitively determine these effects.

Authors' contributions

This study was conducted collaboratively by the authors. Author RA wrote the protocol and performed the statistical analysis. Author MFÖ designed the study, collected the data, and wrote the first draft of the article. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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