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Effect of freezing rate on goat sperm morphology and DNA integrity

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Abstract: This study investigates the effect of freezing rates on the spermatological parameters of frozen and thawed Saanen goat semen. Equilibrated semen was frozen at 4 different freezing rates from +5 °C to –150 °C (G10: 10 °C/min, G12: 12 °C/min, G15: 15 °C/min, and G24: 24 °C/min) and stored in liquid nitrogen until use. Semen samples were examined for sperm motility, defective acrosomes (FITC-PSA), and DNA integrity using TUNEL after dilution with extender A at equilibration and postthaw stage. There was no significant difference among the freezing stages in terms of DNA fragmentation ($P > 0.05$). DNA integrity was partially affected by the freezing rate. The increase of freezing rate from 10 °C/min to 24 °C/min between +5 °C and –150 °C resulted in higher postthaw DNA damage. The study found that the freeze-thawing process is detrimental to postthawed goat semen motility ($P < 0.05$), acrosome integrity ($P < 0.05$), and DNA integrity ($P > 0.05$). Although the freezing rates used in the present study had no effect on postthaw sperm motility and acrosome integrity ($P > 0.05$), sperm DNA integrity was affected.

Key words: DNA integrity, freezing rate, goat semen

1. Introduction

The effects of cryopreservation on sperm function and fertility have been widely studied. Various sperm characteristics (motility, integrity of membrane, DNA function, and mitochondrial function) are known to be affected by the detrimental effects of cryopreservation. As a result, the fertility of frozen and thawed semen is poorer than the fertility of fresh semen (1,2).

The success of cryopreservation depends upon many factors, including interactions among cryoprotectants, type of extender, cooling rate, thawing rate, and packaging, as well as variation among individual animals (1). Amongst the above factors, freezing rate in the critical temperature range is of considerable importance, because the dehydration of spermatozoa is accelerated at slower rates of freezing, whereas more ice crystals form in the spermatozoa at faster rates of freezing (3).

The process of fertilization involves complex biochemical and physiological procedures that cannot be measured solely by routine semen evaluation. The traditional evaluation of ejaculate quality has been based primarily on routine semen analyses (such as motility, morphology, and acrosomal integrity). However, these routine semen evaluations have limited capacity for predicting the potential fertility of the ejaculate (4). Therefore, advanced techniques for semen evaluation (such as in vitro fertilization, cervical mucus penetration, DNA, and plasma membrane integrity) should be implemented to increase the accurate identification of high-quality sperm (5). Several techniques have been proposed to study sperm DNA abnormalities (6). A current technique is the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) technique, which allows for the evaluation of sperm DNA fragmentation (7).

Although many studies have examined the effects of freezing rates on the outcomes of routine spermatological evaluations, few studies have focused on the effects of freezing rates on DNA integrity in mammalian semen. Instead, most of these studies examined human (8,9), ram (10), and boar (11) semen. No studies on goat semen cryopreservation directly evaluated the effects of different freezing rates on semen DNA integrity.

Therefore, the aim of the present study was to compare the effect of different freezing rates on postthaw sperm motility, acrosome integrity, and DNA integrity of frozen and thawed Saanen goat semen.

2. Materials and methods

2.1. Chemicals

Semen dilution buffer chemicals, phosphate buffer solution (PBS) tablets, and poly-L-lysine were purchased from Sigma (Sigma Chemical Co., USA). TritonX-100 (10%

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stock solution) (11332491001) and an in situ cell detection kit were purchased from Roche (Roche Diagnostics GmbH, Germany). Proteinase K (003011) was purchased from Zymed (Zymed, USA). Bovine antirabbit fluorescein (FITC) (sc-2365) and mounting medium (sc-24941) were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology Inc., USA). All other chemicals were purchased from Merck (Merck & Co., Germany).

2.2. Semen collection in animals

This study used 6 Saanen goats aged 3–4 years, with proven fertility, maintained by the Faculty of Veterinary Medicine, Uludağ University, Turkey. Semen was collected from the goats following electrostimulation with an electroejaculator that was standardized for small ruminants (MINITUBE, Germany). Ejaculates were collected twice weekly from the bucks (12). Collection of semen occurred by physically restraining each goat and inserting a lubricated probe into the rectum with downward pressure applied to the front of the probe, so that the electrodes remained near the upper portion of the ampullary region. The electrical stimulation was applied for 4–8 s. When the electrostimulation was stopped briefly, further massage was applied with the probe. This cycle was repeated until 1.5–2 mL of semen was collected (approximately 3–4 electrostimulations). Collected semen was placed in a warm water bath (30 °C) and evaluated immediately for consistency, wave motion (scale of 0–5), and percentage of motile spermatozoa (%) (12).

2.3. Semen dilution, freezing, and thawing

The volume of ejaculates was measured in a conical tube graduated at 0.1-mL intervals. Amongst ejaculates with thick consistency, only 1–2 mL of sperm with rapid wave motion (2–5 on a 0–5 scale) and >70% initial motility was pooled. Five pooled ejaculates were included in the study.

Pooled semen was diluted with a Tris-based extender (20% egg yolk) (v/v) to a final concentration of 1:5 (semen:extender) in 6% glycerol using a 2-step dilution method (12). Briefly, pooled ejaculates were diluted to a ratio of 1:2 (semen:extender) with extender A (no cryoprotectant) and cooled to 5 °C for 1 h. The cooled semen was diluted to a ratio of 1:1 (semen:extender) with extender B (6% glycerol). Extender B was added in 5 steps at 10-min intervals and equilibrated at 5 °C for 2 h. The equilibrated semen samples were frozen in 0.25-mL straws at 4 different freezing rates from +5 °C to 150 °C (G10: 10 °C/min, G12: 12 °C/min, G15: 15 °C/min, and G24: 24 °C/min) in liquid nitrogen vapor using a Nicool Plus PC freezing machine (Air Liquide, France). They were subsequently plunged into liquid nitrogen at –196 °C, where they were stored for at least 1 month. A minimum of 3 straws from each group were thawed at 37 °C for 30 s in a water bath to evaluate postthaw semen characteristics.

2.4. Semen evaluation

All semen parameters were assessed at the following 3 time points: after dilution with extender A, after equilibration, and at thawing. The same researcher performed the freezing of all semen samples, and each studied semen parameter was measured by the same researcher throughout the study. Sperm motility was assessed subjectively using a phase-contrast microscope (Olympus BX 51) (400×) with a warm slide (38 °C).

2.4.1. Fluorescein lectin staining assay (fluorescein isocyanate-conjugated *Pisum sativum* agglutinin (FITC-PSA))

Acrosome integrity was assessed using FITC-conjugated PSA (12). Briefly, 20 mL of diluted semen was resuspended in 500 mL of PBS and centrifuged at 2000 rpm for 20 min; the supernatant was then discarded. The spermatozoa pellet was resuspended in 250 mL of PBS. One drop of resuspended spermatozoa was smeared on a glass microscope slide and air-dried. Air-dried slides were fixed with acetone at 4 °C for 10 min; the slides were then covered with FITC-PSA solution (50 mg/mL in PBS solution) in the dark for 30 min. Stained slides were rinsed with PBS solution, covered with glycerol, and examined under a fluorescence microscope. At least 100 spermatozoa per smear were evaluated for acrosome integrity.

2.4.2. TUNEL assay

For the TUNEL technique, we used an in situ cell death detection kit with fluorescein (Roche Diagnostics) according to the manufacturer's protocol with slight modifications. In brief, one drop of resuspended spermatozoa was smeared on a glass slide and fixed with 10% formaldehyde for 20 min at room temperature. The slides were washed in PBS and stored at 4 °C. Upon removal from storage, the samples were washed again in PBS (3 times for 5 min each). They were then treated in a humidified chamber with proteinase K for 10 min at room temperature, washed with PBS, treated with 3% H_2O_2 in distilled water for 10 min at room temperature, and washed again with PBS. The slides were permeabilized on ice with 0.1% Triton X-100 for 5 min.

The permeabilized slides were incubated in the dark at 37 °C for 1 h with the TUNEL reaction mixture, which contained terminal deoxynucleotidyl transferase (TdT) and dUTP label. After labeling, samples were washed with PBS and analyzed immediately via fluorescence microscopy. Negative (omitting TdT from the reaction mixture) and positive (using DNase I, 1 mg/mL, for 10 min at room temperature) controls were included in each trial. At least 100 sperm were evaluated to determine the percentage of TUNEL-positive sperm. Each microscopic field was evaluated first under fluorescence microscopy (40× magnification) to determine the number of reactive sperm, and then under phase-contrast microscopy to determine the total number of sperm per field.

2.5. Statistical analysis

The study was repeated 5 times, and the results were subjected to statistical analysis with a paired-samples t-test (SPSS 20.0). Differences in confidence values of P < 0.05 were considered to be statistically significant.

3. Results

The effects of different freezing rates on sperm parameters following the freeze-thaw process were evaluated in 5 independent experiments. The Table shows the differences among percentages of motility, defective acrosomes, and DNA fragmentation rates of diluted, equilibrated, and thawed goat semen from different freezing rate groups.

As shown in the Table and the Figure, although postthaw semen motility and acrosome integrity rates in the different freezing rate groups were similar (P > 0.05), sperm motility and acrosome integrity were progressively reduced by the freeze-thaw process (P < 0.05).

There was no significant difference among the freezing stages in terms of DNA fragmentation (P > 0.05), except for G15 and G24 (P < 0.05). DNA fragmentation in G24 (32.5%) was higher than in G15 (19.1%) (P < 0.05). The lowest (G10) and highest (G24) freezing rates led to higher DNA damage compared to the other freezing rate groups.

4. Discussion

The freezing process negatively affects the spermatological parameters of goat spermatozoa, with a similar effect observed in the sperm of other domestic animals. The kidding rate after artificial insemination with frozen and thawed semen is poorer than with fresh or chilled semen (13). There are many studies on optimizing cryopreservation procedures in small ruminants (12,14,15). In the present study, we evaluated the effects of different freezing rates on postthaw semen characteristics and the DNA integrity of frozen goat semen.

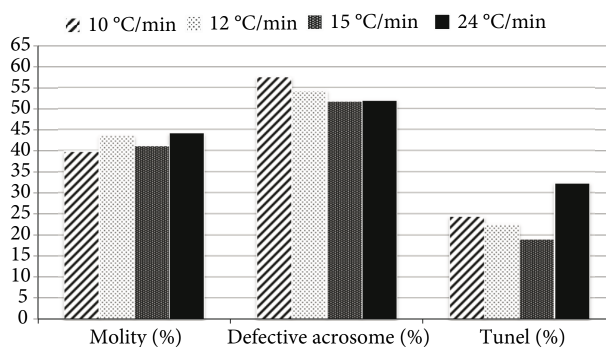

Figure. Postthaw percentages of motility, defective acrosome (FITC-PSA), and DNA fragmentation (TUNEL) rates in different freezing rate groups.

The mean percentages of sperm motility, defective acrosomes, and DNA fragmentation rates in the diluted ejaculates were 71.0%, 25.0%, and 12.0%, respectively. These data are in agreement with previous reports on goat sperm (16,17).

Ultrastructural studies have shown the detrimental effects of cryopreservation on various sperm organelles, including irreversible changes to the acrosomes, mitochondria, and tails of spermatozoa (18). These damages to the mitochondria and tails likely account for the decrease in motility observed after freezing (18). Moreover, swelling of the acrosomal area was observed to be a consequence of cold shock, which indicates a loss of membrane integrity (18).

Table. Mean motility, defective acrosome, and DNA fragmentation rates in diluted, equilibrated, and postthawed Saanen goat semen.

Process	Freezing rates	n	Motility (%), $\overline{X} \pm S\overline{x}$	Defective acrosome, FITC-PSA (%), $\overline{X} \pm S\overline{x}$	DNA fragmentation, TUNEL (%), $\overline{X} \pm S\overline{x}$
Diluted semen	–	5	71.0 ± 1.9^{Aa}	25.0 ± 3.5^{Aa}	12.0 ± 2.8^{Aa}
Equilibrated semen	–	5	57.0 ± 2.0^{Bb}	29.6 ± 1.9^{Aa}	18.0 ± 7.8^{Aab}
Postthawed semen	G10	15	40.0 ± 2.3^{c}	57.6 ± 4.2^{b}	24.4 ± 3.5^{bc}
	G12	15	43.7 ± 1.9^{c}	54.4 ± 3.6^{b}	22.7 ± 3.6^{ab}
	G15	15	41.3 ± 1.6^{c}	51.9 ± 3.7^{b}	19.1 ± 2.8^{ac}
	G24	15	44.3 ± 2.2^{c}	52.1 ± 3.9^{b}	32.5 ± 5.6^{bd}
	Total mean	60	42.3 ± 1.1^{C}	54.0 ± 1.9^{B}	24.7 ± 2.1^{A}

Different superscripts (a, b, c, and d) within the same column for the same stage are significantly different (P < 0.05).
Different superscripts (A, B, and C) within the same column for different stages are significantly different (P < 0.001).
FITC-PSA: Fluorescein isocyanate-conjugated *Pisum sativum* agglutinin.

Freezing and thawing procedures (dilution, equilibration, and thawing) had negative effects on motility and acrosome integrity (P < 0.05). These effects were also observed in the studies of Barbas and Mascarenhas (19) and Dorado et al. (20), which showed that sperm from small ruminants, such as goats, does not have high adaptability to temperature changes, which may contribute to sperm sensitivity**.**

The speed of temperature drop is known to have an effect on the susceptibility of spermatozoa to cold shock and on the quality of sperm after freezing. Generally, cold shock damage manifests as a decline in cell metabolism, altered membrane permeability, loss of intracellular components, irreversible loss of spermatozoon motility, and increase in the number of dead spermatozoa (18). Optimal freezing and thawing rates are critical for developing successful semen cryopreservation protocols. Blanco et al. (21), Bittencourt et al. (22), and Nur et al. (10) reported that freezing rates had no effect on postthaw motility or acrosome defects for poultry, goats, and rams, respectively. Similarly, the 4 freezing rates used in the present study did not affect the motility or the rate of defective acrosomes.

The success of cryopreservation depends upon many factors other than freezing rate, such as species, breed, or variation among individual animals. Different animal species exhibit different sperm membrane compositions, such as different cholesterol/phospholipid ratios and degrees of hydrocarbon chain saturation, which can affect how the sperm responds to cooling and, subsequently, can confer different sperm cryosensitivities across various species (23). Choe et al. (24) reported that rapid freezing was found to be more effective than slow freezing for Korean buck sperm survival. Frankel et al. (25) observed that among the freezing rates of 10, 20, 30, and 40 °C/min, the fastest rate (40 °C/min) yielded significantly better postthaw motility than the slower rates for striped bass sperm.

Martorana et al. (26) reported that slow cooling rates resulted in the least amount of membrane damage and, therefore, in less leakage of cellular components essential to rhesus sperm function. These findings agree with those of Ashrafi et al. (14) and Memon et al. (27), who observed improved values with slower cooling in ram and goat sperm, respectively. Species and breed differences might account for the contradictory results of our study. Additionally, different responses to cooling procedures with respect to sperm characteristics in these studies, compared with the present study, may be due to differences in cooling rates and the organization of the structural components of the sperm plasma membrane.

While no single test accurately predicts the fertility of a sperm sample, a joint examination of various physical characteristics of semen provides more reliable results on potential fertility (28). Therefore, an evaluation of sperm DNA integrity could be useful for assessing the potential fertility of a given sperm sample, when considered along with other semen quality assays. Sperm DNA damage has been associated with poor semen quality (29). The sperm chromatin structure and DNA are known to be altered or damaged during cryopreservation (30). In the present study, the TUNEL results showed that freezing semen caused deterioration in DNA integrity. In addition, chromatin injury in postthaw semen was higher than in diluted and equilibrated spermatozoa.

It is known that damage to DNA integrity might be caused by environmental factors such as elevated temperature, toxic agents, components of semen storage extenders, storage conditions, and the cold shock caused by freezing and thawing. Cold shock increases the susceptibility of semen to oxidative damage due to an increase in reactive oxygen species (ROS) production. ROS have been shown to change cellular functions through the disruption of the sperm plasma membrane and damage to proteins and DNA (26). Hammadeh et al. (8) reported that the freeze-thawing process affected sperm chromatin structure. They also reported that the mean percentage of human spermatozoa with damaged DNA content was higher in semen frozen in liquid nitrogen vapor than in a controlled biological freezer.

The freeze-thawing process is detrimental to postthaw ram semen viability as well as to DNA integrity (12). In the present study, the freeze-thawing process triggered DNA fragmentation to some degree in all groups (P > 0.05). Postthaw ram sperm DNA integrity, as determined by a TUNEL assay, has been shown to be lower in fast-frozen semen than in slow-frozen semen (12). In the present study, comparisons of TUNEL-positive postthaw spermatozoa across the various freezing rates showed that mean values were generally not affected. However, the lowest (G10) and highest (G24) freezing rates led to higher DNA damage compared to other freezing rates. Specifically, DNA fragmentation in G24 (32.5%) was higher than in G15 (19.1%) (P < 0.05).

In conclusion, the freeze-thaw process is detrimental to postthaw goat semen motility (P < 0.05), acrosome integrity (P < 0.05), and DNA integrity (P > 0.05). The freezing rates used in the present study had no effect on sperm motility and acrosome integrity (P > 0.05). DNA integrity was affected by the freezing rate to some degree. The increase of the freezing rate from 10 °C/min to 24 °C/min between +5 °C and –150 °C results in higher postthaw DNA damage.

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