

Effect of freezing rate on acrosome and chromatin integrity in ram semen*

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Summary: The objective of the present study was to investigate the effect of different freezing rates on post-thaw sperm motility, acrosome defect, and sperm chromatin structure and apoptotic activity in ram semen. Collected semen was diluted at 1:5 (semen/extender) with Bioxel® (IMV technologies France) at 30°C and then cooled to 5°C within 1h. Cooled semen was subjected to the equilibration for 2 hours. Equilibrated semen was frozen in 0.25 ml straw at two different cooling rates (slow: 0.5°C/min from 5 to -20°C and fast: 5°C/min from 5 to -20°C). Both groups were frozen from -20 to -120°C at 25°C/min and stored in liquid nitrogen until use. Post-thaw (37°C/30 min) sperm motility, defected acrosome (*Pisum sativum* agglutinin fluorescein conjugate, FITC PSA), sperm chromatin structure determined by Acridin Orange (AO) and apoptotic activity using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) were evaluated. Post-thaw sperm motility, acrosome defect, AO and TUNEL for slow frozen semen were 42.8±8.8%, 31.6±12.9%, 2.9±2.4% and 2.8±1.6%, and for fast frozen semen were 36.5±9.9%, 24.7±11.1%, 3.3±2.2% and 6.3±3.4%, respectively. Post-thaw semen analyses showed that there was no significant difference between two freezing curves in terms of acrosome defect, sperm chromatin damage (AO). However, a significant difference was found for post-thaw semen motility between two groups (P<0.05). In conclusion, while the slow freezing procedure improved post-thaw sperm motility, acrosome and chromatin integrities and apoptotic index in ram spermatozoa did not show any significant difference between freezing rates.

Key words: Apoptosis, freezing rate, ram semen.

Koç spermasında dondurma hızının akrozom ve kromatin bütünlüğü üzerine etkisi

Özet: Bu çalışma, farklı dondurma hızlarının, eritme sonrası motilite, akrozom bütünlüğü, kromatin yapısı ve apoptozis üzerine olan etkilerini araştırmak amacıyla gerçekleştirildi. Toplanan sperma örnekleri 30°C'de 1/5 oranında (sperma/sulandırıcı) Bioxel® (IMV, Fransa) ile sulandırıldı ve 1 saatte +5°C'ye düşürülerek 2 saat süre ile ekilibrasyona bırakıldı. Ekilibre edilen sperma 0.25 ml'lik payetlere çekilerek iki farklı soğutma hızında (yavaş: +5°C'tan -20°C'a 0.5°C/dak ve hızlı: +5°C'tan -20°C'a 5°C/dak) donduruldu. Her iki grup -20°C'tan -120°C'a 25°C/dak hızla donduruldu. Eritme sonrası (37°C/30 dak) aşamada; akrozom bozukluğu *Pisum sativum* agglutinin fluorescein conjugate (FITC PSA) ile kromatin yapısı Acridin Orange (AO) ile ve apoptozis ise terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling tekniği (TUNEL) kullanılarak belirlendi. Motilite muayeneleri ısıtma tablalı faz kontrast mikroskop ile gerçekleştirildi. Eritme sonrası motilite, akrozom bozukluğu, AO ve TUNEL değerleri sırasıyla yavaş dondurma için %42.8±8.8, %31.6±12.9, %2.9±2.4 ve 2.8±1.6 hızlı dondurma için ise %36.5±9.9, %24.7±11.1, %3.3±2.2 ve %6.3±3.4 olarak saptandı. Spermanın eritme sonrası bulgularının değerlendirilmesinde akrozom bozukluğu ve sperma kromatin yapısı (AO) yönünden iki soğutma hızı arasında fark bulunmazken, eritme sonrası motilitede gruplar arasında istatistiksel fark bulundu (P<0.05). Sonuç olarak, koç spermasında yavaş soğutma prosedürü ile dondurma, eritme sonrası motiliteyi iyileştirirken, akrozom ve kromatin bütünlüğünü ve apoptotik indeksi soğutma oranları arasında önemli bir fark göstermemiştir.

Anahtar sözcükler: Apoptozis, dondurma hızı, koç sperması

Introduction

The cryopreservation process of mammalian semen results in lower motility and morphological integrity and higher non-return rate when it was compared with fresh semen (1, 2, 8, 16, 26). Cooling and freezing rates (7, 15,

18, 22, 25), type (5, 16, 25) and concentration (7, 25, 27) of cryoprotective agents (5, 16, 25), dilution rates (15, 18, 20), temperature at which glycerol is added to the semen, equilibration period, and thawing rate interact with the success of post-thaw recovery of spermatozoa

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(1, 2, 7, 19) and fertilizing capability (19, 26). Some authors explain these phenomena with reducing transport ability and survival of ram spermatozoa in the female reproductive tract and loss of viability, morphological and DNA integrity during freezing and thawing procedures (7, 16, 26).

Several researchers have developed different extenders and protocols for freezing ram semen, but the fertility results are not comparable to those obtained with fresh semen and natural mating (7, 18, 22, 25). Thermal shock, formation of ice crystals, dehydration, increased salt concentration and osmotic shock that occur during freeze-thawing have detrimental effects on spermatozoa (22).

The success of cryopreservation of cells is affected by the rate of freezing (10, 11) and the composition of the solution where the cells are frozen (11, 16). When the cells are rapidly cooled, water is not lost fast enough to maintain the equilibrium; the cells become increasingly supercooled and freeze intracellularly, and this process might affect the integrity of chromatin and the morphology of spermatozoa (11).

Sperm DNA damage may occur during spermiogenesis, spermatogenesis, and transport in the female genital tract or during processing (dilution, cooling, freezing and thawing) for freezing by oxidative stress factors that mainly result from reactive oxygen species (ROS) (6). There are several articles in the literature dealing with the effects of freezing rates on post-thaw motility (3), acrosome integrity (21) and fertilizing ability (3) of ram semen. To our knowledge, in the previous studies, there was no direct evaluation of different freezing rates on the chromatin and apoptotic index of ram semen.

Therefore, the aim of the present study was to compare the effect of freezing rates of ram semen on post-thaw sperm motility, acrosome integrity, and sperm chromatin structure index and apoptotic index.

Materials and methods

Chemicals: Tris(hydroxymethyl)aminomethane (THAM), Dextran B (150000-200000), glycerol, 1,2 propanediol, sucrose, trehalose, Acridine Orange (AO), Phosphate buffer solution (PBS) tablets, and poly-L-lysine were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA). Triton X-100 (10% stock solution) (11332481001) and *In Situ* Cell Death Detection Kit were purchased from Roche (Roche Diagnostics GmbH, Mannheim, Germany). Proteinase K (003011) and antibody diluents were purchased from Zymed (Zymed, San Francisco, California, USA). All other chemicals were purchased from Merck (Merck & Co., Inc., Darmstadt, Germany).

Semen Collection and Cryopreservation: Ten Awassi rams, 3-5 years of age, maintained in Uludag University, Faculty of Veterinary Medicine in Bursa, Turkey, were used during the breeding season. Semen was collected by electrically stimulated ejaculation (Ruakura Ram Probe Plastic Products, Hamilton, New Zealand, probe length 12 cm, diameter 2.5 cm, 12 V) five times with a one-day inter-semen collection interval (21, 23). To collect semen, rams were restrained physically and lubricated probe was inserted into the rectum with downward pressure on the front of the probe so the electrodes remained nearby the upper portion of the ampullary region. The electrical stimulation was applied for 4-8 s. While the electrostimulation was stopped briefly (3-4 s), further massage was applied with the probe. This cycle was repeated until 1-2 ml semen was collected (usually 3-4 electrostimulations). Collected semen was placed in a warm water bath (30°C) and evaluated immediately for consistency, wave motion (0-5 scale) and percentage of motile spermatozoa (0-100%) (20). Ejaculates with a thick consistency, rapid wave motion (2-5 on a 0-5 scale) and >70% initial motility were used for freezing. Collected semen were diluted at 1:5 (semen/extender) with Bioxel® (IMV technologies, FRANCE) at 30°C and then cooled to 5°C within 1h and equilibrated for 2 h. The equilibrated semen samples were placed into 0.25 ml straws and the straws from each ram were randomly allocated to one of the two freezing rates (3) slow: 0.5°C/min from 5°C to -20 and fast: 5°C/min from 5°C to -20. Cooling from -20 to -120°C was at 25°C/min. A programmable freezer, Nicool Plus PC freezing machine (Air Liquide, Marne-la-Vallée Cedex 3, France) that had been pre-cooled to 5°C was used. Once straws reached -120°C, they were then plunged into liquid nitrogen at -196°C. At least three straws from each group of frozen ejaculates were thawed in a water bath at 37°C for 30 s to evaluate post-thaw semen characteristics.

Semen evaluation: All semen parameters were assessed after dilution with extender and after thawing. All of the samples were frozen by the same person, and each of the studied semen parameters was measured by the same person on each occasion throughout the study. Sperm motility was assessed subjectively using a phase-contrast microscope (400x) with a heated stage (38°C) (21).

Fluorescein lectin staining assay (FITC-conjugated *Pisum sativum* agglutinin (FITC PSA)): Acrosome integrity was assessed using FITC PSA (12). Briefly, 20 µl of diluted semen was re-suspended in 500 µl PBS and centrifuged at 2000 rpm for 20 min; the supernatant was then discarded. The spermatozoa pellet was resuspended in 250 µl 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, and the slides were covered with FITC PSA solution (50 µg/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.

Acridine-Orange Staining Assay: Sperm DNA integrity was assessed using the AO fluorescence method (11). Air-dried slides were fixed overnight in freshly prepared Carney's solution (three parts of methanol and one part of glacial acetic acid) and allowed to air dry for a few minutes. Dried slides were stained for 3 min with 0.1% AO. The stained slides were evaluated immediately under a fluorescence microscope. Normal DNA content showed green fluorescence over the head region, while DNA abnormalities showed varying fluorescence from yellow-green to red. At least 100 spermatozoa per smear were evaluated for DNA abnormalities (11). Sperm cells with changes in fluorescence from yellow-green to red were recorded as sperm with abnormal DNA content.

TUNEL Assay: For the TUNEL technique, we used the *In Situ* Cell Death Detection Kit with fluorescein (Roche Diagnostics GmbH, Mannheim, Germany) 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, samples were washed again in PBS (three 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% Hydrogen peroxide (H₂O₂) in distilled water for 10 min at room temperature and washed again with PBS. The slides were permeabilized with 0.1% Triton X-100 for 5 min on ice.

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) plus dUTP label. After labeling, samples were washed with PBS and immediately analyzed using 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 cells were evaluated to determine the percentage of TUNEL-positive sperm. Each microscopic field was evaluated first under fluorescence microscopy (40X magnification) to determine the number of reactive sperm and then under phase-contrast microscopy to determine the total number of sperm per field.

Statistical analyses: Data were subjected to a statistical analysis with paired samples T test (SPSS 10.0), and P<0.05 was considered as significant.

Results

Obtained data from each ram were individually analyzed. There were no differences among rams for fresh and post-thaw semen parameters (data not shown). Therefore, obtained semen parameters were pooled and reanalyzed. The mean fresh semen motility and acrosome defect were 73.8, and 5.7%, respectively. Post-thaw semen parameters were summarized in Table 1. Post-thaw semen motility, for slow and fast frozen semen were 42.8% and 36.5%, respectively (P<0.05). FITC PSA was only bound to the acrosomal region of the ram spermatozoa (Figure I). Post-thaw defected acrosome, for slow and fast frozen semen were 31.6% and 24.7%, respectively (P>0.05).

Table 1. The effect of freezing rates on post-thaw semen motility, acrosome and DNA integrity, and apoptotic index
Tablo 1. Dondurma hızının eritme sonrası spermatozoa motilitesi, akrozom ve DNA bütünlüğü, ve apoptotik indeks üzerine etkisi

Freezing rates	Motility	Damaged acrosome	Chromatin index	Apoptotic index
Slow	42.8±8.8 ^a	31.6±12.9 ^a	2.9±2.4 ^a	2.8±1.6 ^a
Fast	36.5±9.9 ^b	24.7±11.1 ^a	3.3±2.2 ^a	6.3±3.4 ^a

^{a,b} Values with different superscripts in the same colon for different freezing rates are different (P<0.05).

FITC PSA: FITC-conjugated *Pisum Sativum* Agglutinin, AO: Acridine Orange

As shown in Figure I (Raw 2), intact DNA of ram spermatozoa showed green fluorescence but the damaged DNA displayed a color spectrum varying from yellow-orange to red. Post-thaw sperm chromatin index (AO staining) for slow and fast frozen semen were 2.9% and 3.3%, respectively (P>0.05).

The TUNEL assay showed that spermatozoa with DNA fragmentation exhibited green fluorescence (Figure I, Raw 3) and undamaged spermatozoa did not show any fluorescence. Post-thaw ram spermatozoa with DNA fragmentation for slow and fast frozen semen were 2.8% and 6.3%, respectively (P>0.05).

The results from post-thaw semen analyses showed that there was no significant difference between two freezing curves in terms of acrosome defect, sperm chromatin damage (AO) and (TUNEL) (P>0.05) but it existed in post-thaw sperm motility (P<0.05).

Discussion

Despite advances in the cryopreservation of bull spermatozoa, there has been lower success with ram spermatozoa (23). Various freezing rates (3), extenders (21)

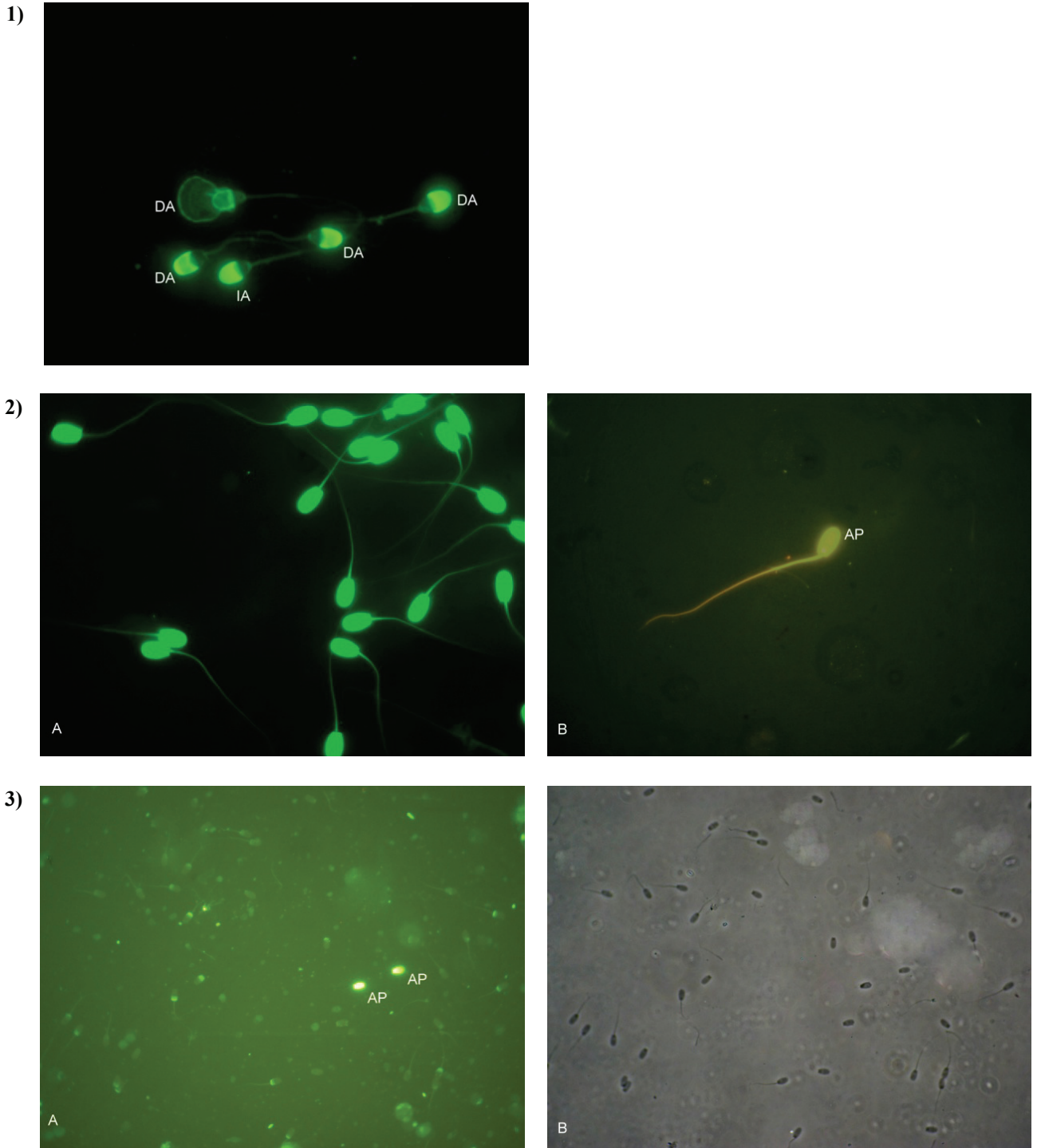


Fig.1. Representative patterns of ram sperm as observed with FITS-PSA, AO and TUNEL staining.

Row 1: FITC-PSA stained spermatozoa with intact and defected acrosome (X40).

Row 2: A: intact, B: fragmented DNA after AO staining (X40).

Row 3: A: Apoptotic spermatozoa under fluorescence microscopy, and B: View under a phase contrast microscopy (X20).

DA: Defected Acrosome, IA: Intact Acrosome, AP: Apoptotic Spermatozoa.

Şekil 1. FITC-PSA, AO ve TUNEL ile boyanmış koç spermatozoasının görüntüleri

Satır 1: FITC-PSA ile boyanmış hasar görmüş akrozom (X40)

Satır 2: A: AO boyama sonrası, A: Sağlam, B: Fragmente (kırık) DNA'lı spermatozoon

Satır 3: A: Apoptotik spermatozoonun floresan mikroskoptaki görünümü, B: Aynı görüntünün faz kontrast mikroskoptaki görünümü.

DA: Hasar görmüş akrozom, IA: Sağlam akrozom, AP: Apoptoza uğramış spermatozoon.

and cryoprotective agents (16, 21) have been described for the cryopreservation of ram semen. Fresh semen motility (73.8%) and acrosome defect (5.7%) were similar to the results of previous studies (8, 16, 21). It is clear that the cryopreservation and/or thawing process is detrimental to sperm viability (14). Slow dehydration of spermatozoa is associated with higher cell survival, whereas in rapid dehydration rates, cell death is more likely (24). Post-thaw motility of slow frozen semen (42.8%) were higher than fast frozen semen (36.5%) ($P < 0.05$). In contrast, O'Neil (17) reported that viability of rapidly frozen semen was better than slow frozen semen.

Freezing and thawing procedures (cooling at 5°C, dilution, equilibration, and thawing) had negative effects on motility ($P < 0.001$) and acrosome integrity ($P < 0.001$) (16). O'Neil (17) reported that semen frozen rapidly (from 5°C to -25°C at 5°C/min) had significantly better acrosome integrity than semen frozen slowly (-0.05°C/min) at the same temperature zone. In contrast, Bryne et al. (3) reported that even though the freezing rates had no statistically important effect on post-thaw acrosome defect, defected acrosome rates of fast frozen semen was higher than slow frozen semen. Similarly, the present study showed that freezing rate has no significant influence on post-thaw acrosome integrity.

While no single test accurately predicts fertility of a sperm sample, examining various physical characteristics of semen together provide more reliable results on potential fertility (9). Therefore, the evaluation of apoptotic index of sperm could be useful together with other semen quality assays to assess the potential fertility of a given sample. The freeze-thaw process is detrimental to post-thaw ram semen viability as well as DNA and functional integrity. (16). TUNEL and AO assays revealed that the freezing rates also caused deterioration of DNA integrity. For TUNEL and AO staining, the mean percentage of thawed spermatozoa with chromatin injury was similar in slow frozen semen to fast frozen semen. Hammedeh et al. (11) reported that freeze-thawing process affected sperm chromatin structure and the mean percentage of human spermatozoa with damaged DNA content were higher in semen frozen on liquid nitrogen vapor than in controlled biological freezer. In the present study post-thaw sperm with chromatin injury in fast and slow frozen samples by TUNEL and AO assay were (6.3% and 3.3%) and (2.8 % and 2.9%), respectively ($P > 0.05$).

In conclusion, while the result of the present study provide clear evidence that freezing rate influence post-thaw semen motility ($P < 0.05$), the acrosome defect ($P > 0.05$), sperm chromatin index ($P > 0.05$) and apoptotic index in ram spermatozoa ($P > 0.05$) were not influenced.

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