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Research Article

Effects of Cooling Rate on Membrane Integrity and Motility Parameters of Cryopreserved Ram Spermatozoa [1]

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Abstract

In this study we aimed to determinate the effects of three different cooling rates from +26°C to +5°C at (0.3°C/min 0.6°C/min and 0.9°C/min) on spermatologic and ultrastructure properties of ram semen. For this purpose semen from 6 rams was collected by electroejaculator and was pooled in a +26°C waterbath. Pooled semen was diluated with tris based extender and divided into three equal parts according cooling rates (0.3°C/min., 0.6°C/min. and 0.9°C/min). Cooled semen was reextended with extender B +5°C in the second step. Diluated samples were equilibrated for 1 h and then were loaded in 0.25 mL straws and freezed in liquid nitrogen vapor. After each freezing stage semen was evaluated motility with computer-assisted semen analysis (CASA). Electron microscobic evaluation was done for pooled and chilled samples. It has been observed that 0.3°C/min. cooled group had meaningfully higher values of motility and progressive motility at +5°C after equilibration and post-thaw stages when compared with the 0.9°C/min. group ($P<0.05$). When compared to the 0.6°C/min., the 0.3°C/min. cooled group had higher total motility values at after cooling to +5°C ($P<0.05$), equilibration ($P<0.05$) and post thaw stages ($P>0.05$) and had higher progressive motility at after cooling to +5°C ($P<0.05$), equilibration ($P>0.05$) and post-thaw stage ($P<0.05$). The TEM evaluation showed that at cooling to the +5°C increases the total damaged spermatozoa in all groups ($P<0.05$). In conclusion, cooling the ram semen to +5°C with a rate above 0.3°C/min. affected negatively the spermatological characteristics. Reaching the cooling rates of 0.6 and 0.9°C/min. increasingly deteriorated the post-thaw motility and progressive motility values. Also, low temperature related to ultrastructural damage was observed at the first dilution step and localized at different regions of the sperm head depends upon the processes and cooling rates.

Keywords: *Ram, Spermatozoa, Cooling rate, Ultrastructure*

Koç Spermasının Dondurulmasında Kullanılan Soğutma Oranlarının Membran Bütünlüğü ve Motilite Özelliklerine Etkisi

Özet

Bu çalışmada koç spermasının 26°C'den +5°C'ye indirilmesinde farklı soğutma hızlarının (0.3°C/dk., 0.6°C/dk. ve 0.9°C/dk.) eritme sonrası spermatolojik özellikler ve spermatozoonların ultrastrüktürel yapısı üzerindeki etkilerinin incelenmesi amaçlanmıştır. Altı adet koçtan elektro ejakülatörle alınan spermalar 26°C'daki su banyosunda pooling işlemine tabii tutuldu. Tris bazlı sulandırıcıyla sulandırılan birleştirilmiş sperma üç eşit hacme bölünerek 3 farklı hızda (0.3, 0.6 ve 0.9°C/dk.) +5°C 'ye soğutuldu. Sperma iki basamakta sulandırıldı, gliserol sperma ısısının +5°C'ye indiği ikinci basamakta katıldı. Sulandırma sonrası sperma 1 saat ekilibre edildi daha sonrasında 0.25 ml payetlere çekilerek sıvı azot buharında dondurulduu. Sperma pooling, sulandırma, soğutma, ekilibrasyon ve eritme sonrası gibi tüm aşamalarında motilite değerleri Bilgisayar Destekli Analiz Sistemleri (CASA) ile değerlendirildi. Pooling ve soğutma sonrasında elektron mikroskop incelemeleri gerçekleştirildi. 0.3°C/dk. soğutma grubunun, spermanın +5°C'ye soğutma, ekilibrasyon ve eritme sonrasındaki hem total motilite hem de progressive motilite değerleri 0.9°C/dk. soğutma grubuna göre önemli derecede yüksek bulundu ($P<0.05$). Bu grup 0.6°C/dk soğutma hızı ile karşılaştırıldığında ise, 0.3°C/dk. soğutma grubunun soğutma ve ekilibrasyon sonrasındaki total motilite değerleri yüksek bulundu ancak eritme sonrası gruplar arasında fark bulunmadı ($P>0.05$). Soğutma ve eritme sonrasında ise progressive motilite değerleri daha yüksek bulunurken($P<0.05$), ekilibrasyon aşamasında progresif motilite değerleri arasında fark bulunmadı($P>0.05$). Yapılan TEM incelemesinde, tüm soğutma hızı gruplarında eritme sonrasında tespit edilen toplam hasarlı spermatozoit oranı, pooling sonrasına göre önemli derecede yüksek bulunmuştur ($P<0.05$). Sonuç olarak koç spermasının dondurulması öncesinde +5°C'ye soğutulmasında 0.3°C/dk.'nın üzerinde soğutma hızlarının kullanılmasının sperma kalitesini olumsuz etkilediği ve soğutma hızı 0.6 ve 0.9°C/dk.'ya arttırıldıkça eritme sonrası total ve progresif motilitenin artan oranlarda etkilendiği sonucu çıkarılmıştır. Ayrıca, koç spermasında düşük sıcaklara bağlı olarak oluşan ultra strüktürel hasarların ilk sulandırma aşamasından itibaren başladığı ve ultra strüktürel hasarların, spermanın gördüğü işleme ve soğutma hızlarına göre başın farklı bölgelerinde lokalize olma eğiliminde olduğu sonucu çıkarılmıştır.

Anahtar sözcükler: *Koç, Spermatozoa, Soğutma oranı, Ultrastrüktür*

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INTRODUCTION

Semen cryopreservation and artificial insemination (AI) have been principal reproductive technologies in cattle industry for so many years. Unfortunately, suboptimal semen preservation methods in combination with the difficulty in passing through the cervix during AI are the major obstacles to the extensive use of cooled or cryopreserved ram semen in AI programs. One of the most integral components of AI programs is semen processing which often requires extenders with ingredients that provide protection to the spermatozoa against cold shock stress [1]. Morphological changes which reduces the fertilization ability of the spermatozoa, occurs during the cooling of spermatozoa and increases during the cryopreservation process [2-4].

Diluted semen is cooled to a temperature close to 0°C. Cooling is a proceses adapts spermatozoa to reduced metabolism. The cooling rate of diluted semen from temperatures above 0°C can significantly influence the post - thaw survival of spermatozoa. Rapid cooling of extended semen from +30 to about +15°C may have no effect on survival of spermatozoa, but fast cooling from +30°C to +10°C, +5°C or 0°C decreases the post-thaw motility of spermatozoa [5]. These damages ultimately can alter functional integrity of spermatozoa and reduces fertililysing ability [3].

Although approximately 40-60% of ram spermatozoa preserve their motility after freeze–thawing, only about 20-30% remains biologically undamaged. Cold shock can harm spermatozoa in various subcellular levels [2,6]. The basic damage to spermatozoa may be ultrastructural (physical), biochemical, functional or DNA integrity [7,8]. Ultrastructural damage occurs to the plasma and acrosome membranes, the acrosome, the mitochondrial sheath and the axoneme. It is well established that ram spermatozoa are relatively more sensitive to cold shock injury than other livestock species' spermatozoa and ultrastructural damage generally is more severe for ram than bull spermatozoa [2,4]. A spermatozoon may be motile, but damaged, in which case it is doubtful if such a cell will fertilise the egg. After both slow and fast freezing of ram semen, motility is better preserved than the morphological integrity of spermatozoa [9].

Any ultrastructural changes like total or acrosomal plazma membrane seperation, partial destruction or fibril defects in the axonemma can only be detected by high resolution Transmission Electron Microscopy (TEM) [10,11].

Early studies showed that cooling spermatozoa to +5°C with high cooling rates negatively affects both the post-thaw sperm quality and motility. However in our knowledge there are no studies on the effects of cooling rates on sperm ultrastructure. The aim of the present study was to determine the effects of different cooling rates (0.3°C/min, 0.6°C/min. and 0.9°C/min.) from +26°C to +5°C on ultrastructure properties and post-thaw motility of ram spermatozoa.

MATERIAL and METHODS

All chemicals used in this study were analytically qualified. Except Ethilen alcohol, Gluteraldehide Merck (Darmstadt, Germany), Osmium Tetraoxide, Proplene Oxide, Epon 812, Uranile Asetate and Lead Asetate, Ladd Research Institute (Vermont USA), all the chemicals were acquired from Sigma Aldrich (St. Louis, MO, USA).

The experiment was performed in accordance with guidelines for animal research from Istanbul University Veterinary Faculty Ethics Commite on Animal Research (2007/183).

Semen Collection

The study was conducted out of the breeding season (May-July). Two to five years old Hemşin rams (n=6) were housed at the Faculty of Veterinary Medicine in Istanbul University under the surveillance of health and nutritional programmes. Ram semen was collected by an electroejeculator (P-T Electronics, Oregon and USA). Implemented electrical stimulatons intervals were 5 second. Semen samples were obtained from all rams following a maximum of 3 or 4 electrical stimuli [12]. The ejaculate was kept in an insulated Styrofoam box containing warm heat pads (30°C) and transported to the laboratory immediately, good quality (volume: ≥0.5 mL; mass motility: ≥4; motility: ≥70%, sperm concentration: ≥2 $\times 10^9$/mL) were pooled [13].

Sperm Collection and Pre-evaluation

A tris-based extender (tris 27.1 g/L, citric acid 14 g/L, fructose 10 g/L, egg yolk 15% (v/v), pH 6.8) was employed to semen. The base extender was divided into two parts and marked as fraction A and B. Then 10% glycerol (v/v) was added to the fraction B, at a final glycerol concentration of 5%. A two-step dilution (with fractions A and B) was used and the glycerol was added in the second step [14]. The pooled semen sample was diluted slowly with fraction A (without glycerol) to final concentration 80×10^6 sperm/ml in a water bath at +26°C. Then, sperm motility and velocity were evaluated by CASA. Then, the pooled semen sample was divided into three equal aliquots (study groups) and cooled to +5°C according to 0.3°C/min, 0.6°C/min., 0.9°C/min cooling rates.

After gliserization and equilibration semen was frozen in liquid nitrogen vapour, 4 cm above the liquid nitrogen level, for 10 min. by using 0.25 mL straws and then were immersed into liquid nitrogen for storage.

Evaluation of Sperm Motility and Kinetic Parameters with CASA System

The pooling, cooling, equilibration and post-thaw motility and kinetic parameters of the sperm samples were measured with computer assissted sperm analysis system (CASA 12.3 IVOS, Hamilton - Thorne Biosciences, Beverly, MA, USA). The sperm analyser was set-up as follows: phase contrast; frame rate – 60 Hz, minimum contrast – 60 Hz, low and high static size gates – 0.6 to 4.32; low and high intensity gates – 0.20 to 1.92; low and high elongation gates 7 to 91; default cell size 5 pixels; default cell intensity-55, VAP (Path Velocity, µm/s), cutoff 20 µm/s, progressive minimum VAP cutoff 50 µm/s, VSL (Progressive Velocity, µm/s) cutoff 30 µm/s. After the CASA system was set to evaluate ram sperm, sperm samples from different stages of cryopreservation process were diluated with tris based diluator and loaded to +37°C heated glass slides (Leja 4, Leja products, Luzernestraat B.V., Holland). Total motility (TM%), progressive motility (PM%), VAP, VSL and other sperm kinetic parameters were measured under 100X magnification and approximetally 600-800 sperm cells in 10 different areas were evaluated [15].

Transmission Electron Microscopy

Pooled and cooled semen were evaluated by the transmission electron microscopy for the ultrastructural defects. The 75 µl of semen from each group were fixated in 0.1 M Phosphate Buffer Saline (pH 7.4) containing 2.5% Gluteraldehide at +4°C, for 4-6 h. After fixation, samples were washed with PBS (pH 7.4) for two times. Then samples were centrifuged at 300 g, for 15 min. and were incubated at +4°C for 24 h. Pellets at the bottom of the tube were transferred to 1% Osmium Tetraoxide (OsO_4) PBS and were incubated at +4°C for 3 h. Following the second incubation peroid, samples were dehidraed through 70%, 80%, 90% and 100% alcohol series for 10 min per each, respectively. Dehidrated samples were washed in Propylene oxide (PPO) for three times for 10 min. to remove alcohol leftovers. Washed samples were blocked by embeding in epon with using Epon Solidication Kit (Fluka Chemie Gmbt, Swiztzerland) in 48 h. Five mm deep bullet shaped blocks were obtained after the blocks were hardened. Sections of 90 nm thickness were cut from those blocks with ultramicrotome (Super Nova Reichert - Yung Austria). Sections were dyed with 3% uranile acetate and lead acetate. Cell membrane distruptions were detected in the sample sections by using JEM – 1010 (Jeol Tokyo) Electron Microscobe and photographed. Differences between groups were evaluated by recording the damages in different areas [16,17].

Statistics

The Kruskal Wallis Test was used for comparation of motility and other kinetic parameters when comparing the cooling rate groups. Mann-Whitney Test was used for evaluation of the ultrastructural findings of the groups after cooling process. In all tests $P<0.05$ was accepted value for statistical significance.

RESULTS

Total and progressive motility values at +5°C of the 0.3°C/min. group was better when compared to 0.6 and 0.9°C/min ($P<0.05$). For the 0.6 and 0.9°C/min. groups motility and progressive motility values were found similar after cooling to +5°C ($P>0.05$). The total and progressive motility of equilibrated spermatozoa of the 0.3°C/min. cooling rate group were significantly higher than 0.9°C/min. rate cooling group's ($P<0.05$). Also the total motility values of the 0.3°C/min. cooling rate were higher than 0.6°C/min. ($P<0.05$) *(Table 1)*. VAP, VSL,VCL, BCF and LIN values were similar in all groups after cooling and equilibration stages ($P>0.05$). Post-thaw 0.3°C/min. group had higher total motility and STR values than 0.9°C/min. group ($P<0.05$). The progressive motility and VCL of the 0.3°C/min. group had ranked significantly higher values than 0.6°C/min. and 0.9°C/min. groups both ($P<0.05$). Moreover the post-thaw VAP values of 0.3°C/min. group was meaningfully higher than 0.6°C/min. group ($P<0.05$) *(Table 2)*.

Such as seperations of the plasma membranes which covers the head in partially or totally were observed in pooled spermatozoa *(Fig. 1A)*. Similar seperations were detected in the cooling groups as were seen in the pooled semen *(Fig. 1 B, C, D)*. In the TEM evaluation, total defected spermatozoa ratio was meaningfully higher in all cooling rate groups when compared to the pooled

Table 1. *Total and progressive motility rates of cooling groups (n= 10)*
Tablo 1. *Soğutma gruplarının toplam ve progresif motilite oranları(n=10)*

Cooling Groups	After Cooling		After Equilibration		Post-Thaw	
	MOT (%)	PMOT (%)	MOT (%)	PMOT (%)	MOT (%)	PMOT (%)
0.3°C/min.	76.1±2.29^{b}	53.3±2.45^{b}	77.1±4.80^{b}	53.9±5.23^{b}	29.8±2.76^{b}	17.8±2.02^{b}
0.6°C/min.	67.8±1.61^{a}	45.3±1.61^{a}	69.0±3.29^{a}	48.8±2.04^{ab}	22.6±2.96^{ab}	11.5±0.99^{a}
0.9°C/min.	60.8±3.32^{a}	40.9±2.92^{a}	68.9±2.16^{a}	44.3±3.21^{a}	16.6±2.94^{a}	10.9±2.30^{a}

^{a,b} *Different superscripts in the same column denote significant differences statistically among Total Motility and Progressive Motility ($P<0.05$);* ***MOT*** *= Total motility,* ***PMOT*** *= Progressive motility*

Table 2. *According to the kinematic values of semen cooling group (n=10)*
Tablo 2. *Soğutma gruplarının sperm kinematik değerleri (n=10)*

Groups		VAP (µm/sn)	VSL (µm/sn)	VCL (µm/sn)	ALH (µm/sn)	BCF (Hz)	STR (%)	LIN (%)
After Cooling	0.3°C/min.	114.3±2.84	100.4±2.74	180.2±2.23	6.3±0.12	40.0±0.91	83.8±0.91	55.2±1.29
	0.6°C/min.	103.7±4.66	90.9±4.62	170.2±5.30	6.3±0.17	39.5±0.85	83.9±1.17	52.9±1.72
	0.9°C/min.	102.4±5.18	90.2±5.30	168.0±6.28	6.1±0.16	40.1±1.18	84.3±1.20	53.5±1.59
After Equilibration	0.3°C/min.	112.1±2.69	97.6±3.14	174.9±1.62	6.1±0.16	40.7±0.96	83.1±1.72	55.6±1.99
	0.6°C/min.	105.7±3.74	93.5±5.06	165.7±5.84	6.0±0.16	40.5±0.93	85.5±1.96	56.5±1.62
	0.9°C/min.	114.0±5.40	99.6±5.11	179.1±4.96	6.3±0.13	42.2±1.21	82.2±2.26	54.8±1.66
Post -Thaw	0.3°C/min.	91.1±2.39^{b}	79.0±2.49	153.1±2.06^{b}	5.7±0.16	41.7±0.62	81.0±1.10	50.8±1.33
	0.6°C/min.	84.9±6.00^{a}	73.8±6.03	147.7±7.97^{a}	6.0±0.15	40.7±0.92	81.5±1.15	49.6±1.21
	0.9°C/min.	88.3±4.23^{ab}	78.4±4.44	145.8±5.66^{a}	5.3±0.14	41.4±0.81	83.9±1.12	53.2±1.67

^{a,b} Different superscripts in the same column denote significant differences statistically among parameters (P<0.05); ***VAP****= Average path velocity,* ***VSL****= Straight linear velocity,* ***VCL****= Curvilinear velocity,* ***ALH****= Lateral head amplitude,* ***BCF****= Beat cross frequency,* ***STR****= Straightness,* ***LIN****= Linearity (Ratio of VSL:VCL)*

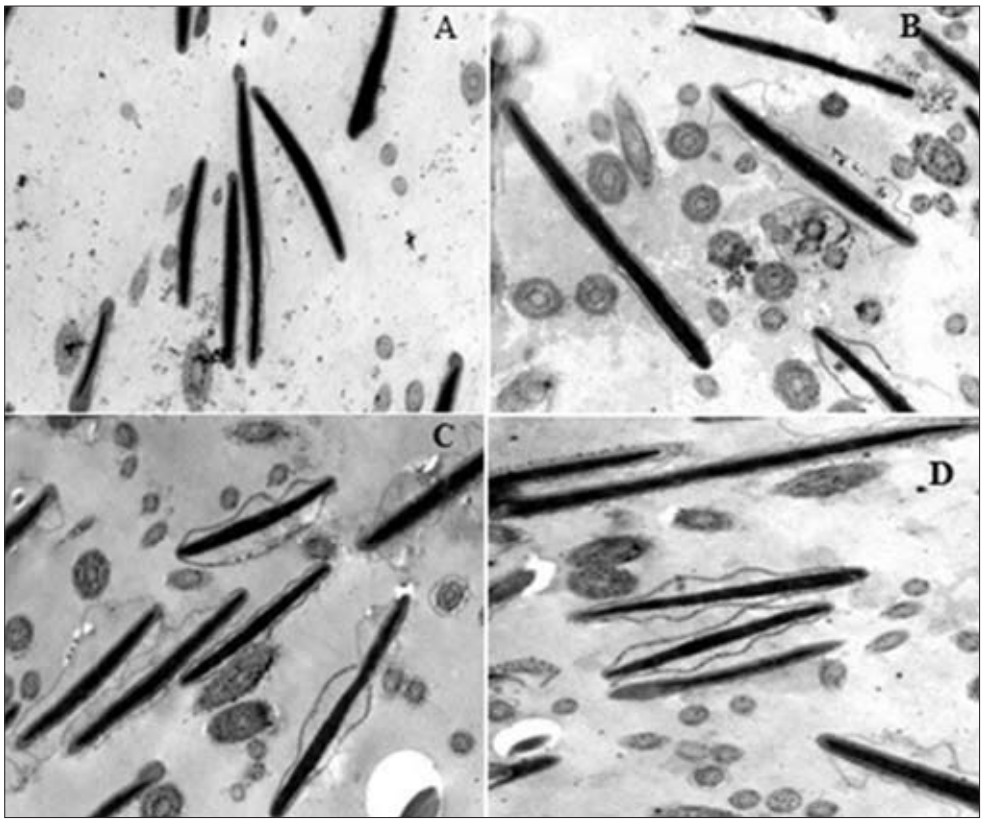

Fig 1. Transmission electron microscopy (TEM) images of Pooling (A) and Cooled (B,C,D) Hemşin ram sperm. In the head of spermatozoa in the plasma membrane ultrastructural changes that occur in different types. Magnification 10.000x

Şekil 1. Pooling (A) ve Soğutma (B, C, D) sonrası Hemşin ırkı koç spermatazoonlarının Transmisyon Elektron Mikroskop (TEM) görüntüleri. Spermatazoonların baş bölgesini saran plazma zarında farklı tipte oluşan ultrastrüktürel değişimler, x10.000 büyütme

semen (P<0.05). This ultrastructural damage in spermatozoa had a tendency to localize at different parts of the spermatozoa according to the cooling rate. When the cooling rate groups were compared with the after pooling stage had lower ratios of ultrastructural damage at the acrosomal region's plasma membrane but on the other hand had higher ratios of damages at the post acrosomal part (P<0.05). The swallowing damage at the plasma membrane covering the acrosome was found higher in 0.3°C/min. cooling group, than the 0.6°C/min. and 0.9°C/min. groups (P<0.05). However in 0.6°C/min and 0.9°C/min. groups, the ultrastructural defects at the whole head region was significantly higher than in the 0.3°C/min. cooling and the pooling stage groups (P<0.05) *(Table 3)*.

DISCUSSION

One of the major causes of reduced motility after freeze thawing is cold shock [18], which often results in swelling and blobbing of the acrosomal membrane and disruption and/or increased permeability of the plasma membrane [9,19]. The morphological damages that occur

Table 3. *In fresh semen (after pooling) and cooled with different cooling rates, ultrastructural damages in spermatozoa that were determined by TEM*
Tablo 3. *Taze (pooling sonrasında) ve farklı soğutma hızları ile soğutulan spermatazoonlarda TEM ile tespit edilen ultrastrüktürel hasarlar*

Groups	Ultrastructural Damages in Cells Based on the Observed Region (%)				
	Post Acrosomal Region	Acrosomal Region	Entire Head	Other Regions	Total Damaged Spermatozoa
Fresh Semen (Pooling)	24[a]	13[a]	20[a]	8[a]	65[c]
0.3°C/dk.	0[b]	54[b]	20[a]	16[a]	90[ab]
0.6°C/dk.	2[b]	28[c]	51[b]	15[a]	96[a]
0.9°C/dk.	2[b]	27[c]	45[b]	12[a]	86[b]

[a,b,c] Different superscripts in the same column denote significant differences statistically among parameters (P<0.05)

at the cell and acrosomal membrane of the spermatozoa during the cooling and freezing processes, reduces the pregnancy rates [20,21]. Cooling the spermatozoa to +15 or +5°C is important, for protecting spermatozoa from cold shock [22]. Dhami et al.[23] have reported post-thaw motility of bull spermatozoa were influenced by cooling rate from +30°C to +5°C. Bacinoglu et al.[24] reported that the detrimental effect of glycerol on post-thaw semen motility was compensated by two step cooling rate regimes. Ak et al.[25] have suggested that, cooling rate from +30°C to +5°C to have controversal effect on semen parameters up to equilibration time but not on post-thaw semen parameters in rams.

In this study the cooling rate of the spermatozoa to +5°C affected both pre and post-thaw quality of the ram spermatozoa. The 0.3°C/min. cooling group had significantly better total and progressive motility values than 0.9°C/min group in cooling to 5°C, equilibration and post-thaw stages. When compared to the 0.6°C/min. group, 0.3°C/min. group had higher total motility values in cooling and equilibration stages and progressive motility values at post-thaw stage (P<0.05). According to the results of the study, 0.6°C/min. cooling rate negatively affected total or progressive motility during different stages of the cryopreservation process, but when the cooling rate was raised to 0.9°C/min in all stages of the process the total and the progressive motility were affected negatively. Similar to our findings, Jones [26] showed that raising the cooling time of the spermatozoa from 30 to 5°C from 1 h to 2 and 3 h, obtained better post-thaw motility (respectively 36.9%, 44.8% and 47.9%, P<0.001).

According to the cooling rate that was used, we have detected morphological ultrastructural changes besides the changes in movement competence of the spermatozoa by evaluating the samples taken from both pre and post cooling stages under TEM. The morphological structural changes occuring during the cooling or freezing of the spermatozoa change the acrosome integrity and also make ultrastructural changes that can not be detected neither under light microscobe nor with flouresance probs [27]. Those morphological changes result lower pregnancy rates at inseminations made with frozen thawed spermatozoa than made with fresh spermatozoa even having the same number of spermatozoa and the same motility values with the frozen thaw samples [9]. The first negative morphological changes in spermatozoa were seen during the gradually cooling stage to 5°C, especially at the outer acrosomal membrane and plasma membrane. Most of the damages occur at the membrane part of the head of the spermatozoa [3,8].

During the cooling stage of spermatozoa due to the temperature changes, lipid molecules in the cell membrane change place with lateral phase transition and these results with destruction of membrane integrity [21]. Fisher and Fairfull [5] reported that there were quite less or non significant destruction was seen in cell membranes when high cooling rate (2°C/min.) were used in ram spermatozoa from 30°C to 15°C. Drobnis et al.[28] indicated that destruction in the spermatozoa cell membrane due to the tempareture began during the cooling stage from 15°C to +5°C. However Holt ve North [29] reported that temperature related to cell membrane changes in the ram spermatozoa as a result of the lateral phase transition due to the temperature change are developed mostly between +17 to +22°C. Wolf et al.[30] declared that the lateral phase transition in cooling the ram spermatozoa occured at 26°C. In this research we have detected that, allthough the motility values have not been affected negatively when pooled at 26°C waterbath, cell membranes had some morphological damages that reduce fertilisation capacity, propably. Most of the morphological damages were seen generally as swelling or seperation of the membrane that covers the head of the spermatozoa, especially at the postacrosomal area, acrosome or the whole membrane itself. Our finding are similar to those Armengol et al.[27], in which detected swelling and seperations at the cell membrane that cover the head part at 30°C after pooling. Detection of 65% cell membrane damage in ram spermatozoa samples pooling indicates that ram spermatozoa are very sensitive to cold damage and these damages can ocur in 26°C in an opposite manner to Drobnis et al.[28].

When the morphological structure changes after

pooling and cooling stages are investigated under electronmicroscope, it is seen that there was a high ratio of swelling and separations at the cell membrane covering the head part. Our electronmicroscopic findings about the ultrastructurel changes in the spermatozoa were similar to the Armengol et al.[27].

We have observed that the ultrastructural damage in spermatozoa was localized in different parts of the head according to the cooling rate. Despite the cell membrane seperations at the acrosome part were the most in the 0.3°C/min. cooling rate group, in the 0.6°C and the 0.9°C/min. groups, these seperations were at the entire cell membrane of the head. The cell membrane that covers the acrosome of the spermatozoa binds with the outer acrosome membrane and reveals the acrosomal enzymes out during the acrosome reaction (AR) while the cell membrane that covers the equatorial segment provides spermatozoa to attach to the oocyte during the fertilisation process. Apart from that, the cell membrane provides to maintain the inner cell ion, pH equilibrium and enzyme activities with its semipermeable structure [10]. The structural integrity of the cell membrane which is at the center of the events occurs synchronously with ovulation like capasitation, acrosome reaction and hypermotility, is irrevocable for fertilisation. The free lipids and proteins that diffuse in between the classical double layered protein/phospholipid structure of the cell membrane are found in the acrosomal, equatorial, postacrosomal, mid piece and last part regions in different concentrations. This differences of dispertion of the lipid mollecules in diferent parts of regional cell membranes cause spermatozoa to show different levels of morphological changes to the cooling rates [31].

In conclusion, it has been investigated out that when ram spermatozoa are cooled to +5°C before cryopreservation, cooling rates above 0.3°C/min. affected the sperm quality negatively and if cooling rates were increased to 0.6 and 0.9°C/min. total and progressive motility at the post-thaw stage was affected with the increasing rate. In this study, we determined that the ultrastructural damages in ram spermatozoa have started to occur at the first dilution stage, and localized at different parts of the head due to the process and cooling rate.

REFERENCES

1. Salamon S, Maxwell WMC: Storage of ram semen. *Anim Reprod Sci*, 62, 77-111 2000.

2. Watson PF: The causes of reduced fertility with cryopreserved semen. *Anim Reprod Sci*, 60-61, 481- 492, 2000.

3. Medeiros CMO, Forell F, Oliveira ATD, Rodrigues JL: Current status of sperm cryopreservation: Why isn't better. *Theriogenology,* 57, 327-344, 2002.

4. Kumar S, Millar JD, Watson PF: The effect of cooling rate on the survival of cryopreserved bull, ram, and boar spermatozoa: A comparison of two controlled-rate cooling machines. *Cryobiology*, 46, 246-253, 2003.

5. Fiser PS, Fairfull RW: The effects of rapid cooling (cold shock) of ram semen, photoperiod, and egg yolk in diluents on the survival of spermatozoa before and after freezing. *Cryobiology,* 23, 599-607, 1986.

6. Yoshida M: Conservation of sperms: Current status and new trends. *Anim Reprod Sci,* 60-61, 121-132, 2000.

7. Akal E, Selçuk M: The factors induced DNA damage of spermatozoon, repair and damage detection mechanisms. *Istanbul Univ Vet Fak Derg,* 39 (2): 284-293, 2013.

8. Holth WV: Fundamental aspects of sperm cryobiology: The importance of species and individual differences. *Theriogenology,* 53, 47-58, 2000.

9. Maxwell WMC, Watson PF: Recent progress in the preservation of ram semen. *Anim Reprod Sci,* 42, 55-65, 1996.

10. Pesch S, Bergmann M: Structure of mammalian spermatozoa in respect to viability, fertility and cryopreservation. *Micron,* 37, 597-612 2006.

11. Zamboni L: The ultrastructural pathology of the spermatozoon as a cause of infertility: The role of electron microscopy in the evaluation of semen quality. *Fertil Steril,* 48, 711- 734, 1987.

12. Nur Z, Zik B, Ustuner B, Sagirkaya H, Ozguden CG: Effects of different cryoprotective agents on ram sperm morphology and DNA integrity. *Theriogenology,* 73, 1267-1275, 2010.

13. Ustüner B, Alçay S, Nur Z, Sağırkaya H, Soylu MK: Effect of egg yolk and soybean lecithin on tris-based extender in post-thaw ram semen quality and *in vitro* fertility. *Kafkas Univ Vet Fak Derg,* 20 (3): 393-398, 2014. DOI: 10.9775/kvfd.2013.10248

14. Cirit U, Bağış H, Demir K, Agca C, Pabuccuoğlu S, Varışlı Ö, Clifford-Rathert C, Agca Y: Comparison of cryoprotective effects of iodixanol, trehalose and cysteamine on ram semen. *Anim Reprod Sci,* 139, 38-44, 2013.

15. Kasimanickam R, Kasimanickam V, Pelzer KD, Dascanio JJ: Effect of breed and sperm concentration on the changes in structural, functional and motility parameters of ram-lamb spermatozoa during storage at 4°C. *Anim Reprod Sci,* 101, 60-73, 2007.

16. Aisen E, Quintana M, Medina V, Morello H, Venturino A: Ultramicroscopic and biochemical changes in ram spermatozoa cryopreserved with trehalose-based hypertonic extenders. *Cryobiology,* 50, 239-249, 2005.

17. Colas C, Junquera C, Perez-Pe R, Cebrıan-Perez JA, Muino-Blanco T: Ultrastructural study of the ability of seminal plasma proteins to protect ram spermatozoa against cold-shock. *Micros Res and Tech*, 72, 566-572, 2009.

18. White IG: Lipids and calcium uptake of sperm in relation to cold shock and preservation: A review. *Reprod Fertil Dev,* 5 (6): 639-658, 1993.

19. Fernández-Santos MR, Esteso MC, Soler AJ, Montoro V, Garde JJ: Effects of egg yolk and cooling rate on the survival of refrigerated red deer *(Cervus elaphus hispanicus)* epididymal spermatozoa. *Reprod Domest Anim,* 41, 114-118, 2006.

20. Anel L, Paz de P, Alvarez M, Chamorro CA, Boixo JC, Manso A, Gonzalez M, Kaabi M, Anel E: Field and *in vitro* assay of three methods for freezing ram semen. *Theriogenology,* 60, 1293-1308, 2003.

21. Bailey JL, Bilodeau JF, Cormier N: Semen cryopreservation in domestic animals: A damaging and capacitating phenomenon. *J Androl,* 21, l-7, 2000.

22. Decuadro-Hansen G: Chilled and frozen semen: the animal experience. *Gyn Obst Fertil,* 32, 887-893, 2004.

23. Dhami AJ, Sahni KL, Mohan G: Effect of various cooling rates (from 30 degrees C to 5 degrees C) and thawing temperatures on the deep-freezing of *Bos taurus* and *Bos bubalis* semen. *Theriogenology,* 38 (3): 565-574, 1992.

24. Bacınoglu S, Cirit U, Nur Z, Ak K: Effect of different glycerol addition techniques and cooling rates on spermatological characteristics in thawed ram semen. *Istanbul Univ Vet Fak Derg,* 33 (1): 11-21, 2007.

25. Ak K, Cirit Ü, Nur Z, Bacınoğlu S, Pabuccuoğlu S, Özdaş ÖB, Birler S: Effects of extender osmolarity, cooling rate, dilution rate and glycerol addition time on post-thaw ram semen characteristics and fertilization.

Istanbul Univ Vet Fak Derg, 36 (2): 33-46, 2010.

26. Jones RC: Influence of diluents and processing times after ejaculation on the survival of deep-frozen ram spermatozoa. *Aust J Biol Sci*, 22 (4): 995-1004, 1969.

27. Armengol MFL, Jurado SB, Pelufo V, Aisen EG: A quantitative ultramorphological approach for systematic assessment of sperm head regions: An example in rams. *Cryobiology,* 64, 223-234, 2012.

28. Drobnis EZ, Crowe LM, Berger T, Anchordoguy TJ, Overstreet SW, Crowe JH: Membrane organization in mammalian spermatozoa cold shock damage is due to lipid phase transitions in cell membranes: A demonstration using sperm as a model. *J Exp Zool,* 265, 432-437, 1993.

29. Holt WV, North RD: Thermotropic phase transitions in the plasma membrane of ram spermatozoa. *J Reprod Fertil,* 78, 447-457, 1986.

30. Wolf DE, Maynard VM, McKinnon CA, Melchior DL: Lipid domains in the ram sperm plasma membrane demonstrated by differential scanning calorimetry. *Proc Nat Acad Sci,* 87, 6893-6896, 1990.

31. Ladha S: Lipid heterogeneity and membrane fluidity in a highly polarized cell, the mammalian spermatozoon. *J Membrane Biol,* 165, 1-10, 1998.