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RESEARCH ARTICLE

The Effect of Different Storage Temperature on Sperm Parameters and DNA Damage in Liquid Stored New Zealand Rabbit Spermatozoa

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Summary

The effect of two different temperatures (4°C and 15°C) on motility, plasma membrane integrity, acrosome abnormality and DNA damage of rabbit spermatozoa was evaluated at 0 and 24 h of liquid storage. Ejaculates collected from six New Zealand male rabbits by artificial vagina and pooled at 37°C following evaluation. Pooled ejaculate was divided into two equal aliquots and diluted with the Tris based semen extender at a final concentration of approximately $40x10^6$ sperms/ml in a Eppendorf plastic tube. There were no significant differences in the percentage of above mentioned parameters between 4°C or 15°C at the beginning of liquid storage (0 h). The percentages of motility (75.0±1.83%) and plasma membrane functional integrity (71.2±1.14%) at 15°C was significantly better than that of liquid stored semen at 4°C (67.9±1.01% and 65.3±1.38%, P<0.05, respectively) at 24 h of storage. The percentage of acrosome abnormality at 24 h wasn't affected by the different storage temperature. The influence of storage temperature and the length of time on spermatozoa DNA damage was found statistically significant (P<0.001). The storage period for up to 24 h lead to an increase in the percentage of spermatozoa DNA damage (P<0.001). The percentages of DNA damage at 4°C was statistically higher than 15°C (P<0.001). In conclusion, 15°C may be prefered when liquid stored rabbit semen are used for 24 h.

Keywords: *Comet assay, DNA integrity, Liquid storage, Rabbit, Semen*

Farklı Saklama Isılarının Kısa Süreli Saklanan Yeni Zelanda Tavşan Spermatozoasının DNA Hasarı ve Sperm Parametreleri Üzerine Etkisi

Özet

İki farklı sıcaklığın (4°C ve 15°C), kısa süreli saklamanın 0. ve 24. saatinde tavşan spermatozoasının motilite, plazma membran bütünlüğü, akrozom anomalisi ve DNA hasarı üzerine etkileri değerlendirildi. Altı Yeni Zelanda erkek tavşandan suni vagina yardımıyla alınan ejakülatlar değerlendirmeyi takiben 37°C'de birleştirildi. Birleştirilmiş ejakülat iki eşit kısıma ayrıldı ve Eppendorf plastik tüpde final yoğunluğu yaklaşık $40x10^6$ sperm/ml olacak şekilde Tris bazlı sperma sulandırıcısı ile sulandırıldı. Kısa süreli saklamanın başlangıcında (0. saat'te) 4°C ve 15°C arasında yukarıda bahsedilen parametrelerin oranında önemli bir farklılık yoktu. Saklamanın 24. saatinde, 15°C'de motilite (%75.0±1.83) ve plazma membran fonksiyonel bütünlüğü oranı (%71.2±1.14), 4°C'de kısa süreli saklanan spermanınkinden (%67.9±1.01 ve %65.3±1.38, P<0.05, sırasıyla) önemli derecede daha iyiydi. 24. saatteki akrozom anormali oranı farklı saklama sıcaklığından etkilenmedi. Sperm DNA hasarına saklama sıcaklığı ve süresinin etkisi istatistiksel olarak anlamlı bulunmuştur (P<0.001). 24 saate kadar süren saklama süresi spermatozoa DNA hasarı oranında artışa neden oldu (P<0.001). 4°C'de DNA hasar oranı 15°C'den istatistiksel olarak daha yüksekti (P<0.001). Sonuç olarak, tavşan spermasının 24 saatlik kısa süreli saklanmasında 15°C tercih edilebilir.

Anahtar sözcükler: *Comet testi, DNA bütünlüğü, Sperma, Tavşan, Kısa süreli saklama*

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INTRODUCTION

Mammalian sperm nuclei are very stable and specifically organises DNA [1]. However, spermatozoa get older due to changes caused storage period at low temperature resulting in sperm plasma membrane deterioration and DNA fragmentation. The integrity of DNA are important aspects of sperm fertility as well routine semen analyses (motility and morphology, functional plasma membrane integrity), because, semen samples may containe high levels of DNA damage are associated with decreased fertility results [2]. The method of comet assay for detecting double-stranded breaks in sperm DNA is a suitable tool for determining damage caused by sperm handling techniques [3,4].

Frozen or liquid stored semen have been widely used to breed cattle by means of artificial insemination [5]. Although, frozen semen is widespread in artificial insemination of cattle, fresh semen generally is preferrred in rabbit breeding. Sperm storage procedures including diluting, cooling, freezing and thawing lead to alteration of sperm biology. According to this alteration, frozen rabbit semen when compared to fresh semen has less motility and viability. Finally, these negatives lead to loss of fertilizing capacity over storage period [6-9].

Storage temperature is an important factor that influences sperm cell function. The temperature below 16°C subject spermatozoa to cold shock [10]. The cold shock induced low temperature can be minimized by suitable semen extenders and optimizing storage temperature and period [11]. In rabbit breeding, liquid stored semen can be used an alternative for artificial insemination. Especially, freshly diluted or cooled semen for a short time may be preferred. In addition, many studies declared that fertilizing capacity of rabbit spermatozoa can be maintained for 2-4 days during liquid storage [12-14].

The aim of this study was to examine the effect of two different temperatures (4°C or 15°C) on motility and plasma membrane functional integrity, acrosome morphology and DNA damage of liquid stored rabbit sperm.

MATERIAL and METHODS

Chemicals

All chemicals used in this study were obtained from Sigma-Aldrich, St. Louis, MO, USA.

Animals, Semen Collection and Semen Processing

Six sexually mature New Zealand white male rabbits were used as semen donors. They were obtained from Experimental and Clinical Research Center of Erciyes University, Kayseri, Turkey. Males were housed in individual cages under standard laboratory conditions (were exposed to a 12 h/12 h light/dark cycle at a room temperature of 22-24°C and 55-60% relative humidity). A commercial pellet diet and fresh drinking water were given *ad libitum*.

Semen was collected two times a week with an artificial vagina from male rabbits. After semen collection, any gel plug was removed. Only ejaculates with good wave motion (≥ 3 on a 0-5 scale) were used. The concentration of spermatozoa was ≥ $300x10^6$ sperm/ml and the percentage of motile sperm was ≥75% were pooled, in order to eliminate individual rabbit differences. A Tris-based extender (313.8 mM Tris, 103.1 mM citric acid and 33.3 mM glucose) containing 5 mg/ml bovine serum albumin (BSA) was used as the basis semen extender. Each pooled ejaculate was divided into two equal aliquots and diluted (at 37°C) with the base semen extender at a final concentration of approximately $40x10^6$ sperm/ml, in a Eppendorf plastic tube. Diluted semen samples were cooled and held at 4°C or 15°C for up to 24 h. Sperm motility, DNA damage, acrosome abnormality and membrane integrity were determined at 4°C or 15°C for periods of 0 and 24 h during liquid storage.

Semen Evaluation

Volume was measured in a graduated conical tube. Motility was assessed at 37°C under light microscope at 100x. Sperm motility estimations were performed in several microscopic fields for each semen sample. The mean of the estimations was recorded as the final motility rate.

The hypoosmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm plasma membrane. HOST relies on the resistance of the membrane to loss of permeability barriers under stress condition of stretching in a hyposmotic medium [15,16]. Sperm cells with resistant membranes exhibited a swelling around the tail such that the flagella become curled and the membrane maintained a swollen 'bubble' around the curled flagellum. The assay was performed by mixing 30 µl of semen with a 300 µl 100 mOsm/kg hypoosmotic solution (9 g fructose plus 4.9 g sodium citrate per liter of distilled water). This mixture was incubated (37°C) for 1 h, where 0.2 ml of the mixture was placed on a microscope slide and mounted with a cover slip and immediately evaluated (magnification x400) under phase-contrast microscope. A total of 200 spermatozoa were counted in at least five different microscopic fields. The percentages of sperm with swollen and curled tails were then recorded.

For the assessment of acrosome abnormality, at least three drops of each sample were added to 1 ml of Hancock solution. One drop of this mixture was put on a slide and covered with a cover slip. The percentage of acrosome abnormality were determined by counting a total of 400 spermatozoa under phase-contrast microscope (magnification x1000 and oil immersion) [17].

Assessment of sperm DNA damage; Diluted semen samples were centrifuged at 300 g for 10 min at 4°C.

Seminal plasma was removed and remaining sperm cells were washed with (Ca^{2+} and Mg^{2+} free) PBS to yield a concentration of $1x10^5$ spermatozoa/cm^3 [18]. Sperm DNA damage was investigated using the single cell gel electrophoresis (comet) assay that was generally performed at high alkaline conditions. Firstly, each microscope slide was pre-coated with a layer of 1% normal melting point agarose in PBS and thoroughly dried at room temperature. Next, 100 µl of 0.7% low melting point agarose at 37°C was mixed with 10 µl of the cell suspension and dropped on top of the first layer. Slides were allowed to solidify for 5 min at 4°C in a moist box. The cover slips were removed and the slides were immersed in freshly prepared cold lysis buffer containing 2.5 M NaCl, 100 mM Na2-EDTA, 10 mM Tris, 1% Triton X-100,10% DMSO and 40 mM dithiothereitol (pH 10) for 1 h at 4°C. Then the slides were incubated overnight at 37°C in 100 µg/ml proteinase K (Sigma) and added to the lysis buffer. The slides were removed from the lysis buffer, drained and placed in a horizontal electrophoresis unit filled with fresh alkaline electrophoresis solution, containing 300 mM NaOH and 1 mM EDTA, (pH 13), for 20 min to allow the DNA to unwind. Electrophoresis was performed for 20 min at 8°C at 12 V and was adjusted to 250 mA. Subsequently, the slides were washed with a neutralizing solution of 0.4 M Tris, pH (7.5), in order to remove alkali and detergents. After neutralization the slides were stained with 50 µl of 2 µl/ml ethidium bromide and covered with a cover slip. All steps were performed under dim light to prevent further DNA damage [19,20]. The images of 50 randomly chosen nuclei were analyzed by CASP [21]. Observations were made at a magnification of 400x using a fluorescent microscope (Olympus, BX51, Japan). Damage was detected by a tail of fragmented DNA that migrated from the sperm head, causing a 'comet' pattern *(Fig. 1)*, whereas whole sperm heads, without a comet, were not considered damaged *(Fig. 2)*. DNA damage was determined by the values of tail DNA damage (%).

Statistical Analysis

Statistical analysis was accomplished with the SPSS system software package (SPSS 15.0 for Windows, 2006; SPSS, Inc., Chicago, IL, USA). The General Linear Models Univariate Analysis of Variance test was utilized to determine the effects of storage temperature and time on rabbit sperm DNA damage during liquid storage. The effects of different storage temperature at 24 h on spermatological parameters were compared by Mann Whitney U test. The results were expressed as the mean ± SEM (Standard Error of Mean).

RESULTS

Analysis of Sperm Parameters (Percentages of Motility, Acrosome Abnormality and Plasma Membrane Functional İntegrity)

The effects of storage temperature at 0 h on motility, acrosome abnormality and membrane integrity are presented in *Table 1*. There were no significant differences in the percentage of above mentioned parameters between 4°C or 15°C at 0 h of storage. On the other hand, the percentages of motility (75.0±1.83%) and plasma membrane functional integrity (71.2±1.14%) at 15°C was significantly better than that of liquid stored semen at 4°C (67.9±1.01% and 65.3±1.38%, $P<0.05$, respectively) at 24 h of storage *(Table 2)*. The percentage of acrosome abnormality at 24 h wasn't affected by the different storage temperature.

Assessment of Sperm DNA Damage

Rabbit sperm DNA damage during different storage temperature (4-15°C) and duration (0-24 h) is also presented in *Table 3*. There were no significant differences in the percentage of sperm DNA damage between 4°C or 15°C at

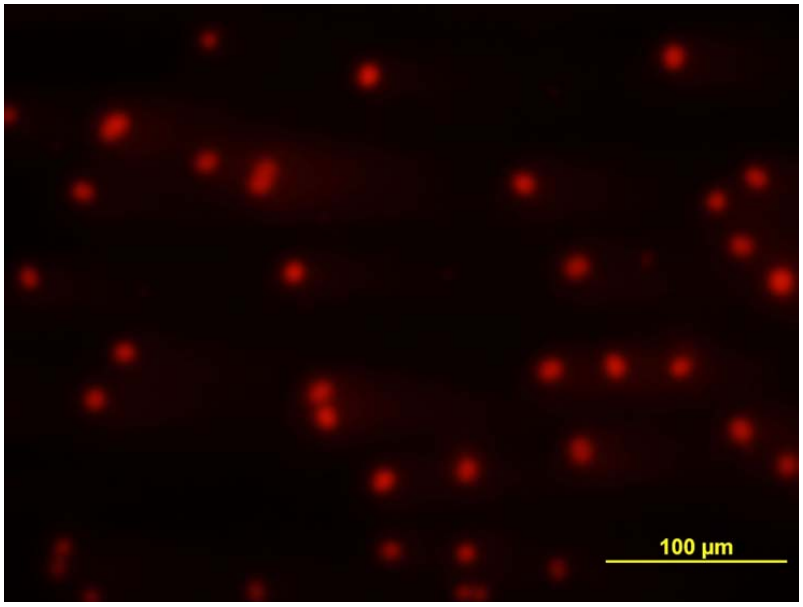

Fig 1. Rabbit sperm cells with a tail of fragmented DNA that migrated from the head. This appearance with a comet pattern shows the sperm with damaged DNA

Şekil 1. Baştan göç etmiş kırık DNA'nın kuyruğu ile tavşan sperm hücreleri. Comet şekilli bu görünüm, hasarlı DNA'lı spermi gösterir

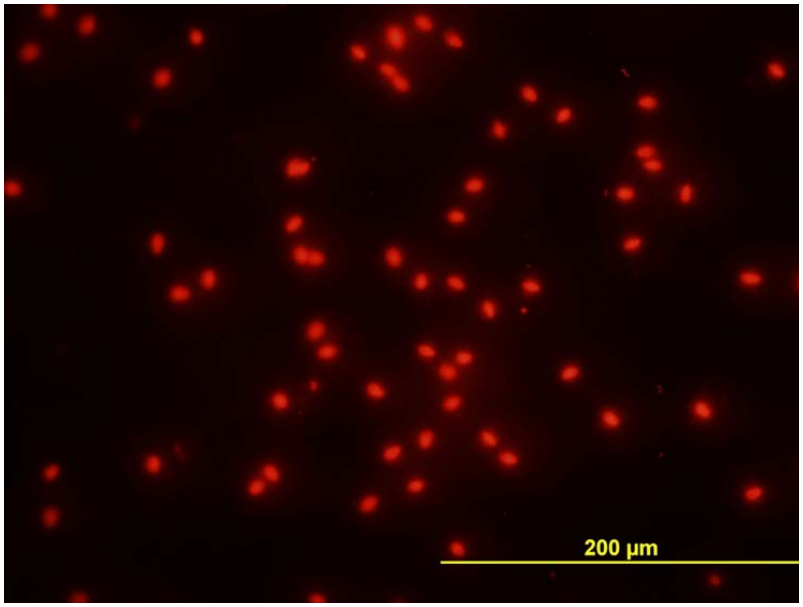

Fig 2. Whole rabbit sperm heads without a comet pattern were considered as undamaged. This appearance without a comet pattern shows the sperm with undamaged DNA

Şekil 2. Comet şekilsiz tam tavşan sperm başları hasarsız olarak kabul edildi. Comet şekilsiz bu görünüm, hasarsız DNA'lı spermi gösterir

Table 1. *Rabbit spermatological parameters (%) at 0 h at the beginning of liquid storage*
Tablo 1. *Kısa süreli saklamanın 0. saatinde tavşan spermatolojik parametreleri (%)*

Parameters	Storage Temperature (C°)	Mean±SEM	Significance
Acrosome abnormality	4	7.7±0.47	P>0.05
	15	7.7±0.56	
Plasma membrane integrity	4	74.9±0.88	P>0.05
	15	76.0±0.86	
Motility	4	82.9±1.01	P>0.05
	15	84.2±0.83	

Table 2. *Rabbit spermatological parameters (%) at 24 h of liquid storage*
Tablo 2. *Kısa süreli saklamanın 24. saatinde tavşan spermatolojik parametreleri (%)*

Parameters	Storage Temperature (C°)	Mean±SEM	Significance
Acrosome abnormality	4	12.1±0.55	P>0.05
	15	11.3±0.67	
Plasma membrane integrity	4	65.3±1.38	P<0.05
	15	71.2±1.14	
Motility	4	67.9±1.01	P<0.05
	15	75.0±1.83	

Table 3. *Tail DNA damage in rabbit semen (%) at 0 and 24 h of liquid storage*
Tablo 3. *Kısa süreli saklamanın 0. ve 24. saatinde tavşan spermasında tail DNA hasarı (%)*

Storage Time (h)	Storage Temperature (C°)	Mean±SEM	Significance (GLM-Univariate)
0	4	1.6±0.35	Time: F: 60.28 P<0.001 Temperature: F: 17.5 P<0.001 Time-Temperature: F: 21.36 P<0.001 R Squared = 0.334
	15	1.8±0.15	
24	4	6.5±0.66	
	15	3.0±0.25	

Time: *Storage Time(h), Independent factor (e.g. Fixed Factor) in the Univariate GLM*
Temperature: *Storage Temperature (C°)), Independent factor in the Univariate GLM*
Time-Temperature: *Interaction between Time and Temperature factors*
F: *F statistic for the degree main effect*
P: *P-value (The observed significance level)*
R Squared: *Coefficient of determination*

the beginning of storage (0 h). The influence of storage temperature and the length of time on spermatozoa DNA damage was found statistically significant (P<0.001). The period for up to 24 h lead to an increase in the percentage of spermatozoa DNA damage (P<0.001). The percentages of DNA damage at 4°C was statistically higher than 15°C (P<0.001).

DISCUSSION

Effective semen processing protocols including extenders, additives, storage temperature and time have been developed to prolonge motility and viability resulting in higher fertility results. Tris-buffer extenders are widely used in many studies that have been found the extender to be beneficial in maintaining sperm characteristics [12,22].

In this study, there were no significant differences in the percentage of motility, acrosome abnormality, plasma membrane integrity and DNA damage between 4°C or 15°C at the beginning of liquid storage. On the other hand, the percentages of motility and plasma membrane functional integrity at 15°C was significantly better than that of liquid stored semen at 4°C at 24 h of storage (P<0.05). The current findings is similar to results obtained by Roca et al.[12] who demostrated that an effective role in maintaining the fertilizing capacity when rabbit semen stored at 15°C up to 72 h.

The semen extender supplemented with BSA showed a protective effect on acrosome morphology of rabbit spermatozoa. The percentage of acrosome abnormality at 24 h was not affected by the different storage temperature. The current findings is similar to results obtained by researchers [23,24] who demostrated that a marked protection in sperm motility, sperm surviving in the reproductive tract of the cow prior to fertilization and the integrity of the plasma membrane and acrosome against cold shock during the freezing-thawing process in semen extender containing BSA.

Cooling temperature has endengered to cold shock and membrane alterations due to phase changes in membrane lipids and osmotic pressure changing [25,26]. The integrity of sperm DNA is very important in maintaining the fertility potential. Various mechanisms can damage sperm DNA. Spermatozoa are particularly susceptible to cold shock-induced damage because their plasma membranes contain large quantities of polyunsaturated fatty acids and their cytoplasm contains low concentrations of scavenging enzymes [27-29].

According to the results of sperm DNA damage that it was affected negatively by the time of storage for up to 24 h. However, 15°C demostrated beneficial role in preserving sperm DNA integrity for up to 24 h. In this study, a significantly negative influence of storage temperature at 4°C was observed on sperm DNA integrity. These findings is in contrast with reported by Rosato and Iaffaldano [30], who 5°C of temperature showed the most beneficial effect in preserving all semen quality parameters during liquid storage of rabbit semen for up to 192 h.

In conclusion, based on the current results, it appeared that 15°C showed significantly beneficial effect on increasing preserving sperm motility and decreasing morphological abnormalities, DNA damages of rabbit spermatozoa aganist chilling injury. The temperature of semen storage is one of the important factors to maintaine motility, DNA structures and stabilizing protein-lipid complex of the sperm membrane. To retain the sperm quality parameters of rabbit spermatozoa is essential chemical composition, suitable cooling temperature and period, handling process that should protect sperm cells from cryogenic injury. Thereby, further studies are needed to developed suitable cooling temperatures, time and influential additives to gain fertile rabbit spermatozoa after cooling process.

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