Kafkas Univ Vet Fak Derg
22 (6): 883-887, 2016
DOI: 10.9775/kvfd.2016.15558

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

# Effect of N-Acetylcysteine (NAC) on Post-thaw Semen Quality of Tushin Rams [1]

Umut Çağln ARI [1] Recai KULAKSIZ [1] Yavuz ÖZTÜRKLER [1]
Necdet Cankat LEHİMCİOĞLU [1] Savaş YILDIZ [1]

[1] *Preliminary results of this study were presented in 19th European Society for Domestic Animal Conference in Varna/Bulgaria (16-19 September 2015)*

[1] Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Kafkas University, TR-36300 Kars - TURKEY

**Article Code: KVFD-2016-15558 Received: 02.03.2016 Accepted: 27.05.2016 Published Online: 27.05.2016**

## Abstract

The aim of the present study was to investigate the effect of N-acetylcysteine (NAC) on freezability of Tushin ram semen. Ejaculates from four Tushin rams were collected with artificial vagina and then pooled. Pooled semen was divided into four aliquots to be diluted with skim milk-based-egg yolk-glycerol (SEG) extender supplemented with various concentrations of NAC (0, 0.25, 0.5 and 0.75 mM). The semen was loaded into 0.25 mL straws, equilibrated (at 4°C for 2 h), frozen in liquid nitrogen (LN) vapour (at -120°C for 15 min) and stored in LN (-196°C). After thawing (at 37°C for 1 min), sperm motility, dead-live ratio, morphology, abnormal acrosome and membrane integrity (HOST) were evaluated. Results showed that 0.75 mM NAC has detrimental effects on motility, compared to the other three NAC doses evaluated (P<0.05). Membrane integrity was higher in 0.25 and 0.5 mM NACs. There was significant differences in semen viability among NAC doses (P<0.05). In conclusion, higher doses of NAC, especially used with SEG extender, may have some detrimental effects on freezability of ram semen. Moreover, although modest doses of NAC slightly improved freezability of Tushin ram semen.

***Keywords:*** *Tushin ram, Semen, Cryopreservation, N-acetylcysteine, Post-thaw semen quality*

# N-Asetilsistein (NAC)'in Dondurulmuş-Çözdürülmüş Tuj Koçu Spermasının Kalitesi Üzerine Etkisi

## Özet

Çalışmanın amacı, Tuj koç spermasının dondurulabilirliği üzerine N-asetil sistein (NAC)'in etkisini araştırmaktı. Dört tuj koçundan suni vajen ile alınan sperma birleştirildi. Karışım sperma dört eşit hacme bölündü ve 0 mM, 0.25 mM, 0.5 mM ve 0.75 mM NAC içeren yağsız süt yumurta sarısı ve gliserollü sulandırıcı ile sulandırıldı. Sperma 0.25 mL'lik payetlere çekildi, ekilibre edidi (4°C'de 2 saat), sıvı azot buharında dondurulduu (-120°C'de 15 dak.) ve sıvı azot (-196°C) içinde saklandı. Çözüm (37°C'de 1 dak.) sonrası, sperm motilitesi, ölü-canlı oranı, morfolojisi, anormal akrozom ve membrane bütünlüğü (HOST) değerlendirildi. Sonuçlar, 0.75 Mm NAC'ın, diğer NAC dozları ile karşılaştırıldığında, motilite üzerine olumsuz etkilere sahip olduğunu gösterdi (P<0.05). Membran bütünlüğü 0.25 ve 0.5 mM NAC derişimlerinde en yüksekti (P<0.05). Ayrıca, NAC dozları arasında, sperma canlılığı açısından önemli farklar da belirlendi (P<0.05). NAC'ın yüksek dozlarının, düşük dozları dondurulabilirliği bir miktar iyileştirse de, özellikle sütlü sulandırıcılarla kullanıldığında, koç spermasının dondurulabilirliği üzerine bazı olumsuz etkileri olabileceği sonucuna varıldı.

***Anahtar sözcükler:*** *Tuj koçu, Sperma, Dondurma, N-asetilsistein, Çözüm sonu sperma kalitesi*

## INTRODUCTION

Tushin sheep breed of the mutton-wool-milk type was bred in Georgia in the 13th-14th centuries under conditions of year-long range husbandry. The breed derives its name from the Tushins, the people of Tushetia, the mountain district where they were developed. According to the breed regionalization plan, the Tushin breed is raised mainly in the Georgian and partly in Azerbaijan, in today's Armenia and in some districts of North East of Turkey (Kars province). Tushin sheep have accommodated to this region hard climate's condition. Its number is estimated as 200.000 and approximately 0.65% of total sheep presence in Turkey [1]. Therefore, this breed can be considered at risk of

**İletişim (Correspondence)**
+90 474 2426800-03/5227
umutari@gmail.com

extinction. Cryopreservation of semen has been considered most important assisted reproductive technique used for protecting extinction breeds. There have been limited number studies carried on cryopreservation and freezability of Tushin ram semen. The reactive oxygen species (ROS: $H_2O_2$, $O_2$, OH, ROOH) and antioxidant defences have been shown to play an important role in fertility and infertility [2,3]. Direct and indirect evidence indicated that some steps of cryopreservation of semen involve the production of toxic ROS [4,5]. Also Griveau and Le Lannou [6] reported that ROS in the ejaculate are produced by the sperm and by leucocytes that infiltrate semen. One of the various ROS, the hydrogen peroxide ($H_2O_2$) decreased sperm motility in many species and it is known that thiols may prevent $H_2O_2$ mediated loss of spermatozoa motility in cryopreserved bull semen [7-9] and also in chilled equine semen [10]. Thiols are a large class of antioxidants that includes cysteine, N-acetylcysteine (NAC), and glutatione (GSH). NAC is precursors of intracellular GSH biosynthesis [11]. There is not any study on Tushin ram semen cryopreservation and also NAC supplemented to milk based extenders in ram semen. Therefore, the aim of the present study was to evaluate the quality of Tushin ram semen after it had been extended and cryopreserved with milk based extender containing different amount of NAC.

## MATERIAL and METHODS

The experiment was carried out according to guidelines for animal research from the National Instutites of Health and all procedures on animals were approved by the Kafkas University Ethic Committee on Animal Research in current study (Approval date/Number: KAÜ-HADYEK 2009/02). All chemicals in current study were provided from Sigma-Aldrich if not stated.

### *Animals*

Four healthy and sexually mature (2 years old) Tushin rams were used in this study. Rams were housed at Education Research and Practice Farm, Faculty of Veterinary Medicine, University of Kafkas Turkey at 40°34'33"N, 43°02'35"E at an altitude of 1751 m. Rams were trained for artificial vagina and were used routinely as semen donors twice a week before the study. They were fed 0.91 kg of concentrate daily, and good quality hay and water were supplied *ad libitum*.

### *Semen Collection*

The four rams were trained to mount a ewe in estrus and serve the artificial vagina. Ejaculates were obtained from each ram by artificial vagina according to the technique previously described by Salamon and Maxwell [12]. Ejaculates were collected twice a week during this experiment. The volume and mass activity were recorded before the tube was placed in a water bath at 37°C. Each ejaculate with 3+ mass activities was analysed to determine its semen concentration, total number of spermatozoa per ml and semen motility, so that adequate semen quality was secured before ejaculates of the two rams were pooled. Only ejaculates with motility >75%, sperm concentration of >2 x $10^9$ spermatozoa/mL and semen volume of >0.5 mL were included in this study. Sperm concentration was determined using hemocytometric method, after diluting semen with Hayem solution (Dilution rate 1/500). The percentage of motile spermatozoa was estimated by subjective microscopic examination using a phase contrast microscope supplied with heated stage at 37°C and magnification 400x after dilution with extender (Dilution rate 1:10).

### *Semen Cryopreservation and Experimental Groups*

The skim milk based extender was all prepared within 1 week period and kept at 5°C. The composition of skim milk based solution was 10 g skim milk powder, 0.9 g glucose, formulated on the basis of 100 mL. 10% (v/v) egg yolk and 5% (v/v) glycerol were added to skim milk based solution. NAC (0 mM, 0.25 mM, 0.5 mM and 0.75 mM) were added to skim milk-egg yolk-glycerol (SEG) extender containing 500 IU of penicillin and 500 µg of streptomycin sulphate per mL. Immediately after collection and evaluation, semen was extended with SEG supplemented with 0 mM, 0.25 mM, 0.5 mM or 0.75 mM NAC. Diluted semen was loaded into 0.25 ml French straws constituting doses of 100 X $10^6$ spermatozoa per straw. Plastic straws were sealed with polyvinyl alcohol powder. The straws were placed in refrigerator at 5°C. The semen was allowed to equilibrate for 2 h before freezing. After equilibration, the straws were frozen horizontally on a rack about 4 cm above liquid nitrogen ($LN_2$) held in an insulated container. The nitrogen vapour reduced the temperature within the straws to -120°C in approximately 15 min. Then the straws were transferred rapidly to $LN_2$ containers at -196°C. The straws were stored in $LN_2$ until evaluation time.

### *Post-thawing Semen Analyses*

After 2 months storage, two straws from each treatment groups (0 mM, 0.25 mM, 0.5 mM, 0.75 mM NAC) were thawed in a warm bath (37°C). After 1 min, the contents of the straw were examined microscopically as described at below. Totally 6 replications were carried out to determine effect of NAC on cryopreservation of ram semen. After thawing of straws, a 3 µL aliquot of each sample was placed on a warmed (37°C) slide and covered with a cover slip before examination under a phase-contrast microscope (Nikon Eclipse E400, Nikon Corp., Japan) at 400x magnification. After observing four or five different fields, the percentage of motile sperm cells was recorded for each sample. Throughout the experiment, two technicians evaluated all the samples without knowing experimental groups and their mean values were recorded as a percentage. For evaluation of acrosomal and morphological abnormalities, one drop sample from groups was diluted in

1 mL Hancock's solution (prepared with 62.5 mL formalin, 150 mL sodium saline solution, 150 mL buffer solution, and 500 mL distilled water [13], placed under a coverslip and evaluated by phase-contrast microscopy (Nikon Eclipse E400, Nikon Corp., Japan) under immersion. The morphological alternations were classified as described by Ax et al.[14]. To evaluate membrane integrity, 50 µL of semen sample was diluted with 450 µL of 100 mM hypotonic solution (composed of 9 g fructose plus 4.9 g sodium citrate per liter of distilled water). After 1 h, smear was prepared and evaluated considering sperm tail curling (%) using bright-field microscopy (Olympus CX21, Olympus Optical Co. Ltd., Japan) (Hypo-osmotic swelling Test/HOST), spermatozoa with curved tail were considered as membrane intact [15]. Percentage of viability in NAC doses after thawing was evaluated with eosin staining (Eosin-Y 1.67 g and sodium citrate 2.9 g dissolved in 100 mL distilled water) as described by Ax et al.[14]. The sperm smears were prepared by mixing a drop of semen with two drops of stain on a warm slide and spreading the stain immediately with the aid of a second slide. The viability was assessed by counting 200 sperm cells with bright-field microscopy (400x) (Olympus CX21, Olympus Optical Co. Ltd., Japan). Sperm showing partial or complete colorization were considered non-viable or dead. Only sperm showing strict exclusion of the stain were considered to be alive [14].

### *Statistical Analysis*

The mean post-thaw semen parameters of motility, dead spermatozoa, abnormal spermatozoa and hypo-osmotic swelling test (curling tail spermatozoa) for the 6 trials carried out during this study were subjected to analysis of variance (One way ANOVA), and differences among means were tested for significance by the Fisher's PLSD. The SPSS 10.0 software was used for all statistical analyses. Differences with values of $P<0.05$ were considered to be statistically significant.

## RESULTS

Results were summarized in *Table 1*. All experimental doses of NAC had similar progressive motility when the results according to six replications were considered to evaluate different doses of NAC, except 0.75 mM NAC (19.1%) ($P<0.05$). 0.25 mM dose of NAC provided lowest percentage of abnormal acrosome (35.2%). Whilst 0.5 mM NAC had highest membrane integrity and viability percentage (43.7 and 34.7% respectively), 0.75 mM NAC had lowest membrane integrity and viability percentages (37.3 and 28.7 respectively), compared with the other doses of NAC ($P<0.05$). Moreover, 0.75 mM NAC had highest dead spermatozoa (lowest viability), abnormal spermatozoa and abnormal acrosome percentages, compared with other groups ($P<0.05$). Briefly, NAC supplementation just improved post-thaw membrane integrity compared with control ($P<0.05$).

## DISCUSSION

In current study, high dose of NAC (0.75mM) especially suppressed the post-thawing motility, while lower doses of NAC there were not any beneficial or detrimental effect on post-thawing sperm parameters, compared with control group. The production of toxic reactive oxygen species, damaging sperm motility and viability [2,16] has been pointed out to be involved in some steps of cryopreservation [5]. Antioxidants such as superoxide dismutase, catalase, cytochrome C and glutathione peroxidase in liquid presentation solutions improved the motility and acrosome integrity of ram spermatozoa, and there was improvement in survival of spermatozoa with increasing dose of antioxidants [17].

Glutathione taking part in Thiols including NAC is one of the most effective protective agents against oxidative damage of bull spermatozoa [18] and also thiols including NAC were demonstrated to prevent hydrogen peroxidase mediated loss of sperm motility in frozen thawed bull semen [8].

The skim milk and the egg yolk contains proteins and lipoproteins shown to have antioxidant properties [19]. However, skim milk and egg yolk contains very few enzymatic antioxidants, especially α-tocopherol, carotenoids, casein and milk proteins [20]. If these non-enzymatic antioxidants are mainly oxidized and not regenerated, this could partly explain why skim milk and egg yolk is not very efficient against oxidative stress and why addition of NAC may have been beneficial to frozen-thawed sperm motility and other parameters in skim milk based egg yolk extender.

***Table 1.*** *Post thaw parameters of Tushin ram semen extended with milk based extender supplemented with different doses of NAC (Mean ± Standard Error)*
***Tablo 1.*** *Farklı dozlarda NAC eklenmiş süt bazlı sulandırıcı ile sulandırılmış Tuj koçu spermasının çözüm sonu parametreleri (Ortalama ± Standart Hata)*

| Concentration of NAC | Motility (%) | Viability (%) | Abnormality (%) | Abnormal Acrosome (%) | Membrane Integrity (%) |
|---|---|---|---|---|---|
| 0.25 mM (n=6) | 26.6±2.4$^{b}$ | 34.4±3.9$^{b}$ | 39.1±1.8$^{b}$ | 35.2±2.0$^{b}$ | 42.6±3.9$^{b}$ |
| 0.5 mM (n=6) | 28.3±4.4$^{b}$ | 34.7±4.0$^{b}$ | 43.9±2.7$^{ab}$ | 38.5±2.0$^{ab}$ | 43.7±2.6$^{b}$ |
| 0.75 mM (n=6) | 19.2±4,6$^{a}$ | 28.7±4,5$^{a}$ | 49.7±4.8$^{a}$ | 44.7±4.5$^{a}$ | 37.3±5.1$^{a}$ |
| 0 mM(Control/n=6) | 28.3±5.2$^{b}$ | 32.3±5.6$^{b}$ | 43.7±4.6$^{b}$ | 37.9±3.4$^{ab}$ | 37.5±4.5$^{a}$ |

$^{a,b,c}$ *Means with different letters differ significantly within the same column ($P<0.05$);* ***n:*** *Number of replication for cryopreservation;* ***NAC:*** *N-acetylcystein*

NAC was added to Tris-glucose-egg yolk extender with high and low doses (0.5 or 1.5 mM) [21-23]. When different doses of NAC were supplemented to Tris egg yolk based extender [21-23] or milk based extender [10], beneficial effects on sperm motility were determined. Although Michael et al.[21,22] determined that supplementation of 1.5 mM NAC to Tris based extender improved sperm motility of frozen-thawed and chilled canine semen, we qualified that higher doses of NAC (i.e. 1 mM and higher) supplemented to skim milk based extender had some directly toxic effects on ram semen, compared low doses of NAC (0.75 mM and lower doses) in our preliminary study [24]. Moreover, in current study, 0.75 mM NAC detrimentally affected motility, viability, morphology and membrane integrity of frozen-thawed ram semen, compared lower doses of NAC groups (0.25 and 0.5 mM) and control (0 mM NAC). The reason of detrimental effect of higher NAC doses may have been originated from acidity of NAC and changing of extender pH. Acidity may cause augmentation and precipitation of milk components especially casein. Also we observed with naked eye that 1 mM and higher doses of NAC supplemented to skim milk based extender caused precipitation and directly toxic effect on ram semen extended with NAC supplemented extender in our preliminary study. Yildiz et al.[24] determined that 5 and 10 mM NAC doses may have toxic effect on ram semen during cryopreservation process. They found that higher NAC doses detrimentally affect sperm motility.

Although there has been limited study [24] about NAC supplementation to skim milk based extender in ram semen, Pagl et al.[10] supplemented 0.2 mM NAC to defined milk protein fraction extender in storage of equine semen at 5°C. They evaluated sperm motility with determined times, they determined that 0.2 mM NAC did not have any detrimental or beneficial effects on sperm motility, compared control group. However, in our current study, it was determined that moderate doses of NAC (0.25 and 0.5 mM) slightly improved sperm viability, morphology, acrosome and membrane integrity. Furthermore, moderate doses of NAC did not show any detrimental or beneficial effect on sperm motility.

Micheal et al.[23] supplemented different NAC doses (0, 0.5, 1, 2.5 or 5 mM) in canine semen extenders and determined that NAC supplementation of semen extenders is beneficial to semen motility of canine spermatozoa during chilling with the 0.5 mM concentration being the most effective, although no significant ROS inhibition was observed at 72 h. It may be said that moderately doses of NAC could improve sperm motility, viability and membrane integrity not only via antioxidant effects but also via other unknown ways.

In conclusion, moderate doses of NAC (i.e. 0.5 mM and lower) supplemented in skim milk based extenders can be used to protect ram sperm cells from oxidative stress without detrimental effect on freeze-ability of ram semen. Furthermore, higher doses of NAC (i.e. 0.75mM and higher), especially used with milk based extender, may have some detrimental effects on freeze-ability of ram semen. It warrants further evaluation in fertility trials.

## Acknowledgements

Authors are grateful to Dr. S.M. Pancarci (University of Balikesir) for his help analyzing the statistic of the manuscript.

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