

Investigation of Ram Sperm Acrosome Integrity in Relation with Seminal Plasma Homocysteine and Nesfatin-1 Levels

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ABSTRACT

This study aimed to investigate the relationship between homocysteine, nesfatin-1 levels with acrosome integrity. Ejaculates were collected from six adult rams via artificial vagina, divided into five groups and diluted at 37 °C. The extenders, which contained cholesterol loaded cyclodextrin (CLC) 1.5 and 2.5 mg, cholesterol loaded 7-dehydrocholesterol (7-DCLC) 1.5 and 2.5 mg or no additive (control), were used for sperm dilution. Semen samples of all groups were cooled at 5°C and then frozen in liquid nitrogen vapor (-110~-120°C). Semen samples stored in liquid nitrogen then were thawed at 38°C for 30 seconds prior to examination. Acrosome integrity was examined with FITC-PNA staining. Homocysteine (HCY) and nesfatin-1 assays were performed with ELISA method. In the CLC 1.5 and 2.5 mg groups homocysteine levels were lower ($0.67\pm 0.11; 0.61\pm 0.26$) compared to control group (1.36 ± 0.9) ($p<0.05$). No statistical differences were observed between groups in nesfatin-1 levels ($p>0.05$). In CLC 2.5 mg, the values of spermatozoa with intact acrosome membrane (65.88 ± 2.84) were higher ($p<0.05$) than in control group (52.38 ± 2.97). Sperm acrosome integrity was negatively correlated with HCY level ($r = -.630$) and positive correlated with nesfatin-1 levels ($r = .460$) in thawed ram sperm. In conclusion, CLC has a cryoprotective effect on acrosome integrity and found to have beneficial effects on HCY level.

Keywords: Acrosome integrity, Cyclodextrin, 7-dehydrocholesterol, Homocysteine, Nesfatin-1

Koç Sperma Akrozom Bütünlüğünün Seminal Plazma Homosistein ve Nesfatin-1 Düzeyleri ile İlişkili Olarak İncelenmesi

ÖZ

Bu çalışmada homosistein, nesfatin-1 düzeyleri ile akrozom bütünlüğü arasındaki ilişkinin araştırılması amaçlanmıştır. Ejakülatlar, suni vajen yöntemiyle altı yetişkin koçtan alındı ve toplanan ejakülatlar beş gruba bölünerek 37 °C'de sulandırıldı. Sperma sulandırılmasında kolesterol yüklü siklodekstrin (CLC) 1.5 ve 2.5 mg, kolesterol yüklü 7-dehidrokolesterol (7-DCLC) 1.5 ve 2.5 mg içeren veya katkı maddesi içermeyen (kontrol) sulandırıcılar kullanıldı. Tüm gruplardaki sperma örnekleri 5 °C de soğutulduktan sonra sıvı nitrojen buharında (-110~-120° C) donduruldu. Sıvı nitrojen içinde saklanan sperma örnekleri incelenmek için 38 °C ve 30 saniyede çözündürüldü. Akrozom bütünlüğü, FITC-PNA boyama ile incelendi. Homosistein (HCY) ve nesfatin-1 testleri ELISA yöntemi ile yapıldı. CLC 1.5 ve 2.5 mg gruplarında homosistein seviyeleri kontrol grubuna (1.36 ± 0.9) göre daha düşük bulundu ($0.67\pm 0.11, 0.61\pm 0.26$) ($p < 0.05$). Nesfatin-1 düzeylerinde gruplar arasında istatistiksel olarak fark gözlenmedi ($p > 0.05$). 2.5 mg CLC'de akrozom bütünlüğü bozulmamış sperma hücrelerinin oranları (65.88 ± 2.84) kontrol grubuna (52.38 ± 2.97) göre daha yüksek bulundu ($p < 0.05$). Sperma akrozom bütünlüğü, çözüm sonu koç spermasında HCY seviyesi ($r = -.630$) ile negatif, nesfatin-1 seviyeleri ($r = .460$) ile pozitif korelasyon gösterdi. Sonuç olarak, CLC'nin akrozom bütünlüğü üzerinde kriyoprotektif bir etki gösterdiği ve HCY seviyesi üzerinde faydalı olduğu belirlenmiştir.

Anahtar Kelimeler: Akrozom bütünlüğü, Siklodekstrin, 7-dehidrokolesterol, Homosistein, Nesfatin-1

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INTRODUCTION

Artificial insemination plays an important role in sheep breeding. Breeds with superior genetic characteristics can be grown with the application of this tool (Maxwell and Watson 1996). The lipid composition of plasma membrane of ram sperm is different compared to the other species (Yurdakök et al. 2015). Consequently, ram sperm cannot be frozen successfully as bull sperm (Salamon and Maxwell 2000). The lipid components of the spermatozoa membranes affect the ability of sperm to respond on freezing, as well as the ability of sperm to provide the acrosome reaction (Moore et al. 2005). The plasma membrane and outer acrosomal membrane of the sperm cell are very sensitive to the freeze-thaw process. These structures, consisting of thermodynamic phospholipids (65-70%) and pass irreversibly from the liquid phase to the gel phase when the membranes cool down (Watson 2000). The cholesterol-phospholipid ratio in the sperm plasma membranes changes during the freezing of the sperm, resulting in reduced fertility (Khan et al. 2017).

Cyclodextrin (CLC) is cyclic heptasaccharides consisting of β -(1-4) water-soluble glycopyranose units. CLC is used to add cholesterol to the cell membrane or to extract from the cell membrane (Purdy and Graham 2004). CLC is cholesterol-like hydrophobic compounds and carry the cholesterol of the cell plasma membrane into the hydrophobic region (Zidovetzki and Levitan 2007). When CLC is used in sperm cells, it ensures the removal of cholesterol that causes capacitation (Mocé et al. 2010). Researchers have demonstrated the ability to cholesterol loaded CLC in different species to increase sperm cryoprotection in stallion (Blommaert et al. 2016), ram (Naseer et al. 2015) and dog (Khan et al. 2017). Cholesterol is an important structural component of the membrane and takes part in the regulation of membrane function (Yeagle 1985). Cholesterol has many effects on the plasma membrane property. While reducing membrane permeability and phase changes, it provides a suitable environment for proteins and it is also a membrane antioxidant (Aksoy et al. 2010; Mocé et al. 2010). Cholesterol provides the transition temperature of the membranes and keeps them liquid, this feature allows it to reduce membrane damage (Glazar et al. 2009).

Homocysteine (HCY) is an amino acid produced by intracellular demethylation of methionine in methylation processes. The high level of HCY in the cell reveals its pathological effects by increasing oxidative stress (Voutilainen et al. 1999). Defective methylation, activation of apoptosis, induction of oxidative stress, altered nitric oxide bioavailability and increased inflammatory cytokine expression are molecular mechanisms of cellular dysfunction caused by HCY (Forges et al. 2007). High HCY concentration,

abnormal spermatogenesis and infertility were found to coexist in male animals (Kelly et al. 2005)

Nesfatin-1 (N1) is an anorexigenic peptide involved in nutrition and metabolic regulation. It is reported that N1 is found not only in the brain, but also in peripheral organs such as digestive organs, heart and reproductive organs (Kim and Yang 2012). N1 is co-localized with gonadotropin releasing hormone, so it has been reported to be involved in gonadotropin release (García-Galiano et al. 2012). Studies have shown the important role of N1 in regulating male reproductive functions (Gao et al. 2016, Ranjan et al. 2019). N1 is localized with oxytocin (Maejima et al. 2009), a neurohormone associated with reproduction and erection. It is stated that the in vitro administration of N1 has a positive role in the regulation of reproduction in male rats (Gao et al. 2016).

In this study, we evaluated the effect of CLC and cholesterol loaded 7-dehydrocholesterol (7-DCLC) modification on the freezing of merino ram sperm by determining acrosome integrity and aimed to investigate its relationship between HCY and N1 levels.

MATERIALS and METHODS

Animals and Semen Collection

Sperm samples were collected from six adult merino rams with an artificial vagina. Semen collection procedures involving the use of animals were performed at Bahri Dagdas International Agricultural Research Institute. The study was approved by Institute Ethics Committee (No 2016/51).

Semen Processing

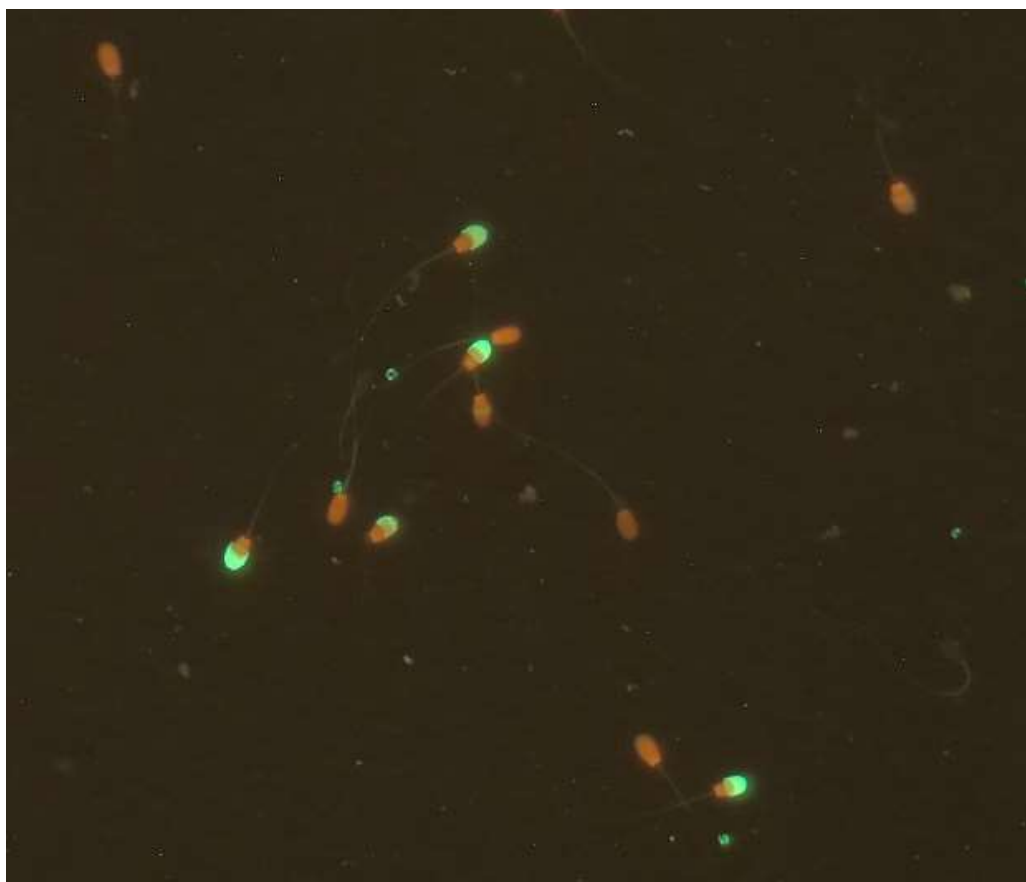
Tris Stock (T) extender (297.58 mM tris, 82.66 mM fructose, 96.32 mM citric acid, 15% egg yolk, 6% glycerol) was used as the main sperm extender in the study. Collected ejaculates (spermatozoa with >80% motility and concentration higher than 2×10^9 spermatozoa/ml) after mixing, the ejaculate was divided into five aliquots and diluted at 37 °C with base extenders containing CLC (1.5 and 2.5 mg), 7-DCLC (1.5 and 2.5 mg) and no additive) diluents (control). Semen samples were equilibrated at 5°C for 2 hours, and then loaded into 0.25 ml straws. Samples were frozen 5 cm above liquid nitrogen vapor for 12 minutes. Then stored in liquid nitrogen (-196°C). Straws were thawed after a one month later in a water bath at 38°C for 30 seconds and evaluated.

Assessment of Sperm Acrosome Integrity

Acrosome integrity was determined the method by Nagy et al. (2003). Thawed semen samples were diluted (1:3 with T) then 60 μ l were mixed with 10 μ l FITC-PNA and 2.5 μ l PI. Samples were incubated in dark at 37° C for 20 minutes. Sperm acrosome integrity was determined using a fluorescence microscope (Carl Zeiss Axioscope 5 GmbH 07745, Germany). Sperm cells displaying bright green fluorescence were

considered damaged, while those in red color in the acrosome cover that did not stain green fluorescence were considered intact.

Figure 1: Lectin/PI staining. Green headed shows damaged acrosome and red headed spermatozoon shows intact acrosome.



based on biotin double antibody technology (Shangai Sunred, Biological Tech., China). After the procedure

Semen Preparation for Enzyme Assays

To separate the cells from diluted seminal plasma, thawed semen samples were centrifuged at 4 °C and 800 rpm for 15 minutes. All samples were washed twice with phosphate buffered saline (PBS, ph 7.2, tablet/200 mL) after centrifugation, the supernatant was removed then pellet was completed to 500 µl with PBS. The prepared sperm suspension was sonicated with a probe for 10 seconds on ice.

HCY Assays

HCY peptides were blindly measured and read (450 nm) ELISA plate reader (ELx800 Absorbance Microplate Reader) by the Biotin double-antibody technology (Shangai Sunred, Biological Tech., China). Homocysteine concentrations were calculated from standard curves.

N1 Assays

N1 measurement was performed by commercial enzyme-dependent immune sorbent assay (ELISA)

of the ELISA kit, plates were read at 450 nm by the ELISA plate reader (ELx800 Absorbance Microplate Reader-Biotech).

Statistical Analysis

The normality and homogeneity of variances were checked with the help of the Shapiro-Wilk test. One Way Anova was used to evaluate sperm results and the results were expressed as mean \pm standard deviation. Analysis of variance, followed by Duncan's post hoc test to identify differences between groups. Differences with $p < 0.05$ values were considered statistically significant. SPSS 21 package program was used for analysis.

RESULTS

In 1.5 and 2.5 mg CLC groups, the values of spermatozoa with intact acrosome membranes (63.04% \pm 2.19% and 65.88% \pm 2.84% for 1.5 and 2.5

mg CLC groups, respectively) were statistically different ($p < 0.05$) compared to the control group (52.38 ± 2.97 ; Table 1). No statistical differences ($p < 0.05$) were found between the two 7-DCLC groups and the control group. Levels of homocysteine were lower ($p < 0.05$) in 1.5 mg CLC (0.67 ± 0.11) and 2.5 mg CLC (0.61 ± 0.26) compared to the control group

(1.36 ± 0.9 ; Table 1). There was no statistical difference between the experimental groups about nesfatin-1 ($p > 0.05$; Table 1). Furthermore, acrosome integrity of thawed ram semen was negatively correlated with HCY ($r = -.630$) and positively with N1 ($r = .460$; Table 2).

Table 1. Mean (\pm SEM) HCY, N1 levels and acrosome integrity in thawed ram semen.

Groups	HCY(μ mol/L)	N1(ng/ml)	Acrosome integrity (%)
Control	1.36 ± 0.9^a	1.71 ± 0.16	52.38 ± 2.97^{bc}
CLC 1.5 mg	0.67 ± 0.11^b	2.12 ± 0.39	63.04 ± 2.19^a
CLC 2.5 mg	0.61 ± 0.26^b	2.30 ± 0.91	65.88 ± 2.84^a
7-DCLC 1.5mg	0.85 ± 0.40^{ab}	1.78 ± 0.14	55.26 ± 1.44^b
7-DCLC 2.5 mg	1.06 ± 0.51^a	2.08 ± 0.25	51.76 ± 0.69^c
P	*	-	*

–: No significant difference.

* ($P < 0.05$).

Table 2. Correlation results in thawed ram semen.

		Acrosome integrity	HCY	N1
Acrosome integrity	Pearson Correlation	1	$-.630^{**}$	$.460^*$
	Sig. (2-tailed)		,001	,021
	N	25	25	25
HCY	Pearson Correlation	$-.630^{**}$	1	$-.408^*$
	Sig. (2-tailed)	,001		,043
	N	25	25	25
N1	Pearson Correlation	$.460^*$	$-.408^*$	1
	Sig. (2-tailed)	,021	,043	
	N	25	25	25

** . Correlation is significant at the 0.01 level (2-tailed).

* . Correlation is significant at the 0.05 level (2-tailed).

DISCUSSION

The highest acrosome integrity rate was obtained in the group supplemented with CLC and a negative correlation was determined between acrosome reacted spermatozoa and the HCY level. It was seen that the determined negative correlation with HCY level could be directly related to sperm freezing. Cholesterol shows its effect on the cell membrane by protecting the antioxidative enzymes and preventing their spread to reactive oxidative species (López-Revuelta et al. 2007). This information supports our conclusions

regarding the membrane integrity of cholesterol forms. In the cryopreservation of bull semen with CLC, it was provided a higher sperm membrane integrity after thawing (Purdy and Graham 2004). Positive result was obtained in the stallion semen as in the presented study (Moraes et al. 2015). Some researchers have found that the use of CLC conjugates on Piau swine (Pinho et al. 2016) and boar sperm (Tomás et al. 2011) has no protective effect on sperm parameters. This difference between the results of the studies is thought to be due to the dose difference.

In CLC-treated sperm, high cholesterol concentrations before cooling and low cholesterol loss during cryopreservation can be achieved. However, the presence of excess cholesterol in the plasma membrane can adversely affect its capacitation and acrosome reaction (Purdy and Graham 2004). Supplementing the ram sperm diluent with cholesterol conjugates and 7-DCLC has been shown to increase membrane integrity (Inanc et al. 2018). Amorim et al. (2009) stated that cholesterol or pelargonic used in freezing bull semen is beneficial for thawed sperm membrane. In the presented study, the highest acrosome integrity values were reached in CLC groups. Although the effect of HCY on the male reproductive system is not fully explained, there is a positive correlation between the increase in seminal plasma HCY level and the decrease in spermatological parameters (Wallock et al. 2001). It is thought that HCY creates its pathological effect by inducing oxidative stress, which explains the etiology of the metabolite in defective sperm function. (Sibrian-Vazquez et al. 2010). HCY and glutathione (GSH) concentrations in the sperm cell were significantly lower in normozoospermic men and increased in those with pathological sperm parameters (Kralikova et al. 2017). These results are consistent with the negative correlation with the acrosome integrity we presented in our study. The increase in acrosome integrity observed in humans (Renard et al. 1996) and stallion (Trimeche et al. 1999) as a result of minimizing the level of lipid peroxidation and it is consistent with the decreased homocysteine level with increased acrosome integrity observed in this study.

N1 is the 82-amino acid hormone important in the regulation of reproduction derived from nucleobindin 2 precursor protein (Gao et al. 2016). It was localized in leydig cells in the testicles. N1 levels in testicles of adult rats were found to be higher compared to pubertal rats (García-Galiano et al. 2012). Tamer et al. (2018) showed that N1 protects spermatogenic cells by providing pro-inflammatory / anti-inflammatory cytokine balance in rats. N1 induced enhanced energy substrate transport may be responsible for promoting spermatogenesis. N1 significantly reduced oxidative stress by its stimulating effect on testicular activities. Thus, it is stated that nesfatin-1 positively affects spermatological parameters. Ranjan et al. (2019) reported that nesfatin-1 increased sperm count and fertility by increasing testicular function. In our study, it was positively correlated with acrosome integrity.

CONCLUSIONS

Adding CLC in semen extender had a protective effect on freezing ram semen. HCY and N1 levels were found to have an effect on semen freezing, and it was suggested that it would be useful to investigate spermatological parameters and HCY and N1 levels together in future studies.

Ethics Committee Information: The study was approved by Institute Ethics Committee (Bahri Dagdas International Agricultural Research Institute, No 2016/51).

Conflict of interest: The authors declare that there is no actual, potential or perceived conflict of interest for this article.

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