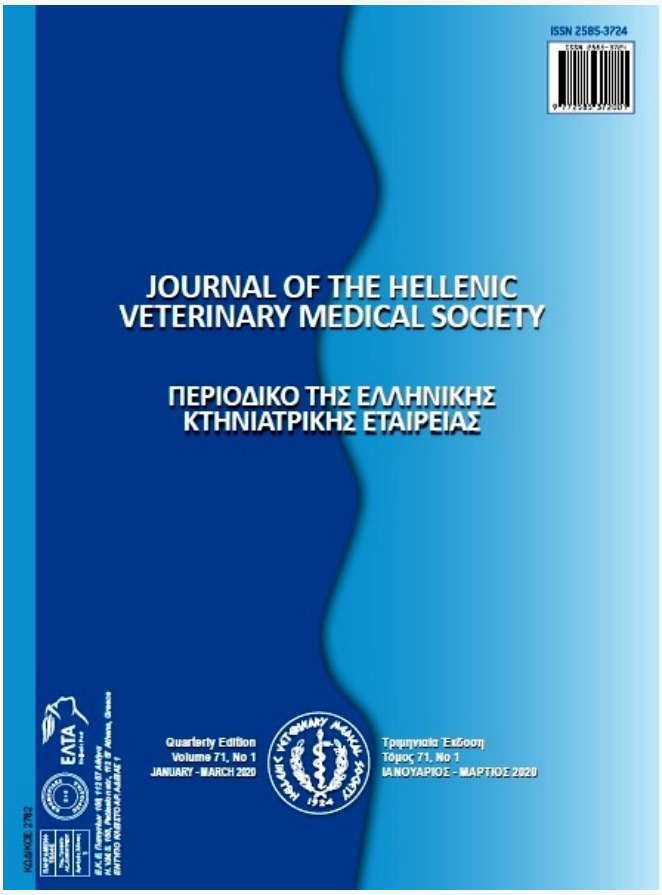
Journal of the Hellenic Veterinary Medical Society

Vol 71, No 1 (2020)



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doi: 10.12681/jhvms.22968

To cite this article:

KÜÇÜK, N., & AKSOY, M. (2020). Effect of environmental heat stress on Kıvırcık ram sperm parameters. *Journal of the Hellenic Veterinary Medical Society*, *71*(1), 2073–2080. https://doi.org/10.12681/jhvms.22968

J HELLENIC VET MED SOC 2020, 71(1): 2073-2080
ΠΕΚΕ 2020, 71(1): 2073-2080

Research article
Ερευνητικό άρθρο

Effect of environmental heat stress on *Kıvırcık* ram sperm parameters

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ABSTRACT. The relationship between environmental conditions and reproduction has been in sight of research. Therefore, there has been a growing interest in effect of climate change, global warming and subsequently environmental heat stress on gametes in last decades. Thus, the present study was designed to investigate the effect of environmental heat stress on *Kıvırcık* ram sperm parameters. In this study, semen samples *(n*=72) were collected from six mature rams during a 12-month study period. The temperature and relative humidity of animal box were controlled daily. The mean monthly temperature humidity index (THI) values were calculated to determine heat stress and control periods. According to the mean monthly THI values, the 36 of 72 ejaculates, which were collected between May and October, were classified as heat stress period samples. The others (*n*=36), which were collected between November and April, were considered as control period samples. The heat stress period sperm samples had lower sperm concentration, motility, viability, membrane integrity and higher abnormal sperm rate compared to control period sperm samples ($P < 0.05$). Semen volume, sperm DNA integrity and the ability of sperm to undergo acrosome reaction were detected similar between the heat stress and control periods. In conclusion, environmental heat stress was found deleterious for some sperm parameters in *Kıvırcık* rams.

Keywords: Heat stress; Ram sperm; Membrane integrity; DNA integrity; Acrosome reaction.

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Date of initial submission: 12-09-2019
Date of revised submission: 24-10-2019
Date of acceptance: 27-11-2019

INTRODUCTION

Stress is revealed by the inability of a living organism to cope with environmental conditions (Dobson and Smith, 2000). Living organisms are influenced by various environmental stress factors such as heat. In last decades, the effect of environmental heat stress on reproduction of wild and domestic animals has received greater attention because of global climate change and global warming. Heat stress essentially causes over production of reactive oxygen species (ROS) and induces oxidative stress (Slimen et al., 2014). The exaggerated production of ROS impairs the physiology and functions of different type of cells by damaging lipids, proteins, polysaccharides and DNA (Cross et al., 1987). Although controlled production of ROS plays important role in sperm functions, uncontrolled production of ROS causes sperm pathology (De Lamirande et al., 1997). Thus, different techniques have been developed to investigate the effect of heat stress on male fertility [e.g., insulation of the entire scrotum or neck of the scrotum (Arman et al., 2006; Brito et al., 2003; Kastelic et al., 1996), immersion of the scrotum in a hot water bath (Banks et al., 2005; Rockett et al., 2001), surgically induced cryptorchidism (Yin et al., 1997) and artificially heated animal boxes (Meyerhoeffer et al., 1985) or naturally hot places (Al-Ghetaa, 2012; Nichi et al., 2006). Previous studies have shown that heat stress affects testicular and epididymal sperm cells (Banks et al., 2005; Karabinus et al., 1997; Sailer et al., 1997) furthermore the spermatocytes and early spermatids are the highly vulnerable germ cells against to heat stress (Rockett et al., 2001; Setchell, 2006). Artificially heating of testes decreases sperm DNA integrity (Banks et al., 2005), semen concentration, sperm motility and fertilizing capacity of sperm in rodents (Jannes et al., 1998). Moreover, similar induction of heat stress increases apoptosis in the germ cells and alters the expression of some genes in the testis (Rockett et al., 2001) and epididymis (Li et al., 2008).

In farm animals, artificially local heating of testes decreases sperm motility (Arman et al., 2006), damages sperm plasma and acrosome membranes (Hamilton et al., 2016) as well as chromatin of the epididymal and testicular sperm cells (Karabinus et al., 1997). The environmental heat stress increases abnormal sperm rate in bulls (Nichi et al., 2006) and elevates dead and abnormal sperm rate in sheep (Al-Ghetaa, 2012). Previous studies indicated that sperm quality gradually returns normal levels within 8 weeks in bulls (Meyerhoeffer et al., 1985) and within 47 days in rams after heat stress (Hamilton et al., 2016). Mostly the previous studies related to the heat stress focused on effect of artificially local heating of testes on sperm parameters. Moreover, the studies based on environmental heat stress were generally investigated the effect of heat stress on basic sperm parameters (motility, viability and morphological abnormalities). Thus, the present study was designed to provide further information about the effect of environmental heat stress on *Kıvırcık* ram sperm parameters including sperm plasma membrane, DNA integrity and ability of sperm to undergo acrosome reaction.

MATERIALS AND METHODS

Animal care and management

Six mature *Kıvırcık* rams were used in this study. The *Kıvırcık* is common breed of the region where the present study was conducted. The animals were housed in an animal box at the Department of Reproduction and Artificial Insemination, Aydın Adnan Menderes University, Turkey. The animals were submitted to uniform nutritional conditions in 15 months. The first 3 months were considered an adaptation period in which the animals adjust to the new feeding and housing system. Permission for use of the experimental rams was obtained from the ethical committee of our university (2012/44).

Calculation of temperature humidity index

The study was conducted in Aydın (approximately 37° north, 27° east and 64 m above sea level) Turkey, where temperatures can reach 45°C under the sun in summer months. The temperature and relative humidity of animal box was controlled daily. The daily temperature humidity index (THI) was calculated with the formula that was previously reported by Marai et al. (2007). In the formula below, T and RH represent respectively temperature (°C) and relative humidity (%).

$$\text{THI} = T - (0.31 - 0.0031 \times RH) \times (T - 14.4)$$

Determination of heat stress and control periods

The mean monthly THI values were used to distinguish heat stress and control periods and to determine the level of heat stress (Table 2) according to the previously defined heat stress categories for sheep by Marai et al. (2007) (Table 1).

Table 1. Definition of heat stress categories according to THI values by Marai et al. (2007).

THI values	Heat stress category
THI < 22.2	Absence of heat stress
22.2 ≤ THI < 23.3	Moderate heat stress
23.3 ≤ THI < 25.6	Severe heat stress
THI ≥ 25.6	Extreme severe heat stress

Table 2. Definition of heat stress categories in heat stress and control periods according to mean monthly THI values.

Periods	Months	Mean THI ± SEM.	Heat stress category
Heat stress period	May	22.3 ± 0.32	Moderate heat stress
	June	24.6 ± 0.27	Severe heat stress
	July	27.1 ± 0.19	Extreme severe heat stress
	August	28.1 ± 0.21	Extreme severe heat stress
	September	22.6 ± 0.22	Moderate heat stress
	October	17.9 ± 0.38	Absence of heat stress (Prolonged effect of heat stress)
Control period	November	15.6 ± 0.27	Absence of heat stress
	December	10.2 ± 0.44	Absence of heat stress
	January	13.0 ± 0.34	Absence of heat stress
	February	13.6 ± 0.29	Absence of heat stress
	March	15.0 ± 0.27	Absence of heat stress
	April	18.2 ± 0.29	Absence of heat stress

Semen collection and evaluation

Semen was collected from six mature *Kıvırcık* rams using an electro-ejaculator at 15-day intervals throughout the study period (12 months). A total of 72 ejaculates (12 ejaculates per ram) were evaluated immediately after collection. Semen volumes and concentrations were determined using a micropipette and a haemocytometer respectively. Motility parameter was evaluated subjectively under a phase contrast microscope at 200× magnification (TMS Nikon, Tokyo, Japan), equipped with a heated stage adjusted to 37 °C using an 8 µl sample of diluted semen [1:5 with Tris, citric acid, glucose (TCG) solution including 300 mM Tris, 95 mM citric acid monohydrate and 28 mM glucose, pH 7.0]. Sperm viability was determined using the eosin-nigrosin (EN) staining procedure. Briefly, diluted sperm samples (5 µl) were stained with EN stain (1% eosin, 3% sodium citrate and 3% nigrosin in distilled water) and thin, uniform smears were made. After air dried, 200 sperm from each sample were observed for live (unstained) or dead (stained) sperm heads under bright field microscopy at 400× magnification. The morphologically abnormal sperm rate was detected with a wet-mount slide method. Briefly, 10 µl semen samples were added in 1 ml of Hancock's solution (Hancock, 1952). A total of 200 sperm from each sample were counted for morphological abnormalities under a phase-contrast microscope at ×1000 magnification.

Assessment of sperm plasma membrane integrity

Sperm plasma membrane integrity was evaluated with hypo-osmotic swelling test in combination with an EN staining procedure (Aksoy et al., 2008). Briefly, 25 µl sperm samples were mixed to 475 µl of fructose solution (100 mOsm/L). The mixed samples were incubated in 35°C water bath for 15 min. Following incubation, sperm samples were stained with EN stain and sperm plasma membrane integrity was assessed with under bright field microscope at 400× magnification. A total of 200 sperm from each sample were scored for numbers of live-intact and total intact (respectively, unstained heads of sperm with coiled tails and stained and unstained heads of sperm with coiled tails).

Assessment of sperm DNA integrity

Sperm DNA integrity was analyzed with comet assay procedure as reported previously (Küçük et al., 2014). Briefly, the slides were covered with 1% normal-melting-point agarose in phosphate buffered saline (PBS). A 10 µl aliquot of diluted sperm extended

in 190 µl 0.75% low-melting-point agarose in PBS final concentration of 2 x 10^4 sperm was added to each slide, covered with a coverslip and cooled for 10 min at 4°C to solidify. After solidification, coverslip were removed gently. The slides were immersed in an alkaline lysis solution (1 M Tris–HCl, 1% N-lauroylsarcosine, 0.5 M EDTA, 0.3 M mercaptoethanol pH >10) at 4°C overnight for the lysis process. The slides were then washed with distilled water to remove lysis solution. The slides were placed in the alkaline electrophoresis solution including 1 mM EDTA and 300 mM NaOH for equilibration (20 min) and electrophoresis (50 V for 40 min). Subsequently, the slides were washed with neutralizing solution of 0.4 M Tris (pH 7) and then fixed with an alcohol series (50%, 70% and 90%). The nucleus of sperm cells were stained with DAPI (40,6-diamidino-2-phenyindole, dilactate) and covered with coverslips. The images of one-hundred randomly chosen sperm nucleus have intact or non-intact chromatin were immediately taken under fluorescent microscope (QuickCam Pro). The images were evaluated with Image J software (version 1.47v; NIH. USA) (Pic. 1) to determine DNA fragmented sperm rate.

Assessment of the ability of sperm to undergo acrosome reaction

Acrosome reacted sperm rate was determined with a Coomassie Blue G-250 staining procedure (Ahmad et al., 2013) in fresh semen, at 0 min after 1 h incubation period, 20, 40 and 60 min after lysophosphatidylcholine (LPC) treatment, respectively. In fresh semen, a 15 µl sperm sample was directly fixed. Another 200 µl portion of fresh sperm was diluted in 2 ml TCG and centrifugation was applied twice at 800× g for 5 min to remove seminal plasma. The remaining sperm pellets were diluted in 1 ml TCG and incubated in a water bath at 35°C for 1 h to induce capacitation. Following incubation, the sperm samples were treated with LPC, an acrosome reaction-inducing agent, as described previously (Ahmad et al., 2013). Briefly, 980 µl sperm sample was supplemented with 20 µl of LPC from a stock solution (10 mg/ml in TCG). The final LPC concentration was 200 µg/ml. The sperm samples were treated with LPC at 35°C for 20, 40 and 60 min. A total of 200 spermatozoa from each sample were observed with bright field microscopy at 400× magnification to detect whether they had visible acrosome (intact) or had invisible acrosome (reacted).

Statistical analyses

All the results collected from present study were analyzed with a Statistical Package (Minitab version 17 Statistical Software). Descriptive statistics were used to determine the mean monthly temperature and humidity index. Two-sample *t*-test was applied to compare the sperm parameters (semen volume, concentration, motility, percentage of live, abnormal, live-intact, total-intact, DNA damaged and acrosome reacted sperm rates) between the heat stress and control periods. A probability level of $P < 0.05$ was used to define statistical significance. All results were presented as mean ± SEM.

RESULTS

Estimation of heat stress and control periods

The mean monthly THI values and severity of heat stress are presented in Table 2. The five months (from May to September), when THI values higher than 22.2 (Marai et al., 2007), were classified as heat stress period. Since detrimental effects of heat stress disappear gradually within approximately one spermatogenesis cycle (47 days) in rams (Hamilton et al., 2016) October was also added in heat stress period (May to October). Remaining six months (from November to April) when the THI values were lower than 22.2, were classified as control period (Table 2).

Evaluation of semen parameters

There was no difference ($P > 0.05$) in semen volume between the heat stress and control periods (Figure 1). Sperm concentration was lower ($P < 0.05$) in heat stress period compared to the control period (Figure 2). Moreover, heat stress decreased ($P < 0.05$) sperm motility, live sperm rate and increased abnormal sperm rate (Figure 3). Heat stress also decreased sperm plasma membrane integrity. Total-intact sperm rate was lower ($P < 0.05$) in the heat stress period compared to the control period (Figure 3). Live-intact sperm rate was lower in the heat stress period compared to control period but statistical difference was $P = 0.06$. DNA damaged sperm rates were found similar in the heat stress and control periods (Figure 3). LPC induced acrosome reacted sperm rate were also found similar in both periods (Figure 4).

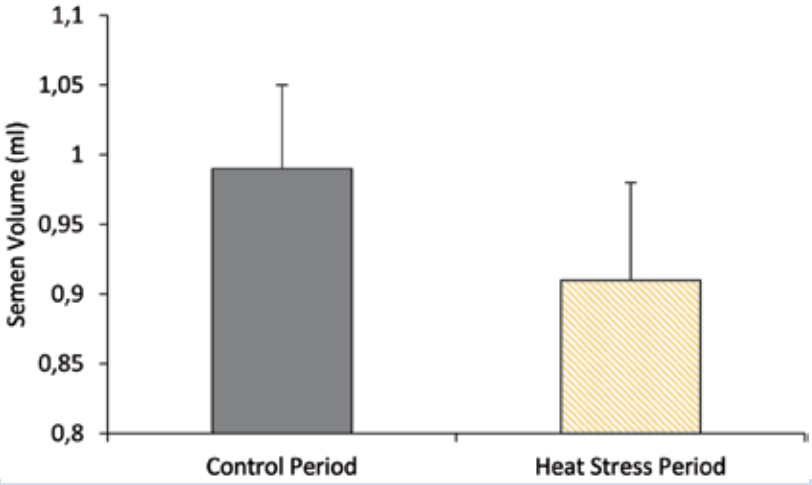

Figure 1. The effect of heat stress on semen volume in rams (P > 0.05).

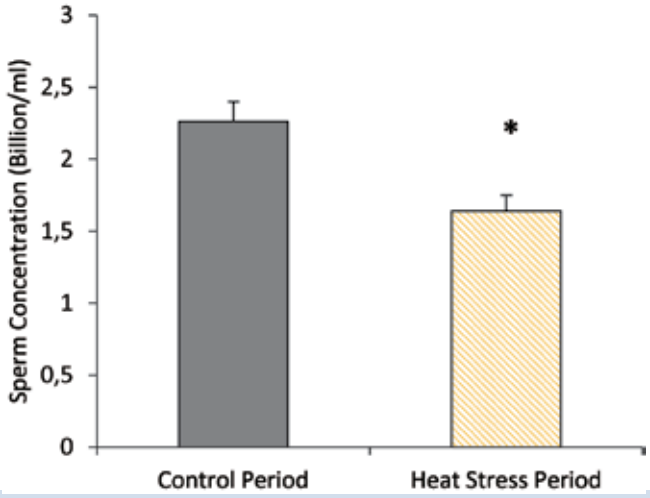

Figure 2. Effect of heat stress on sperm concentration in rams. Asterisks indicate P < 0.05.

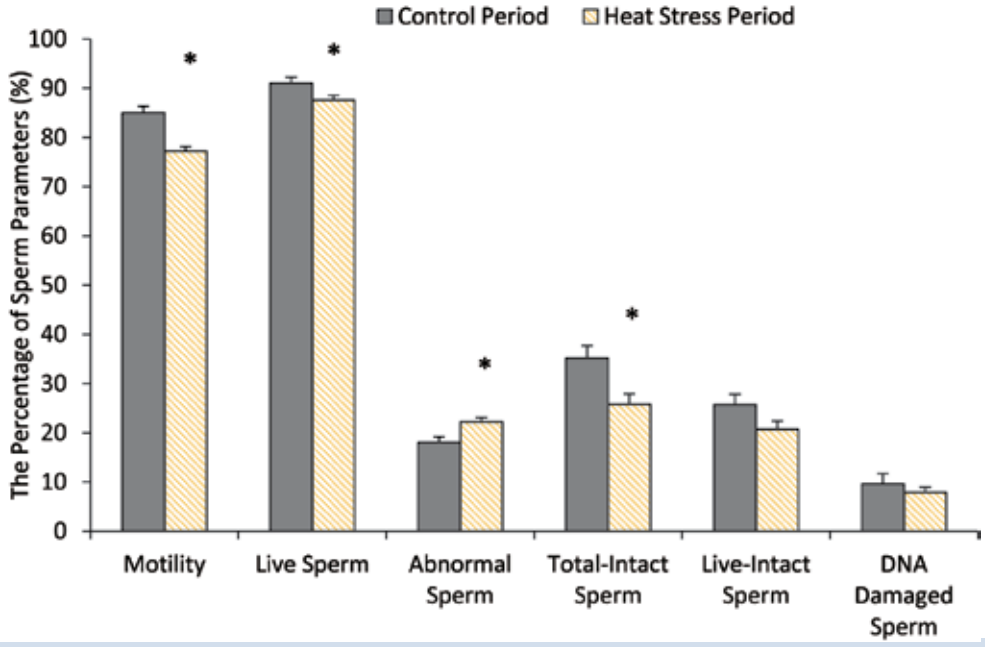

Figure 3. Effect of heat stress on in vitro sperm parameters in rams. Asterisks indicate P < 0.05.

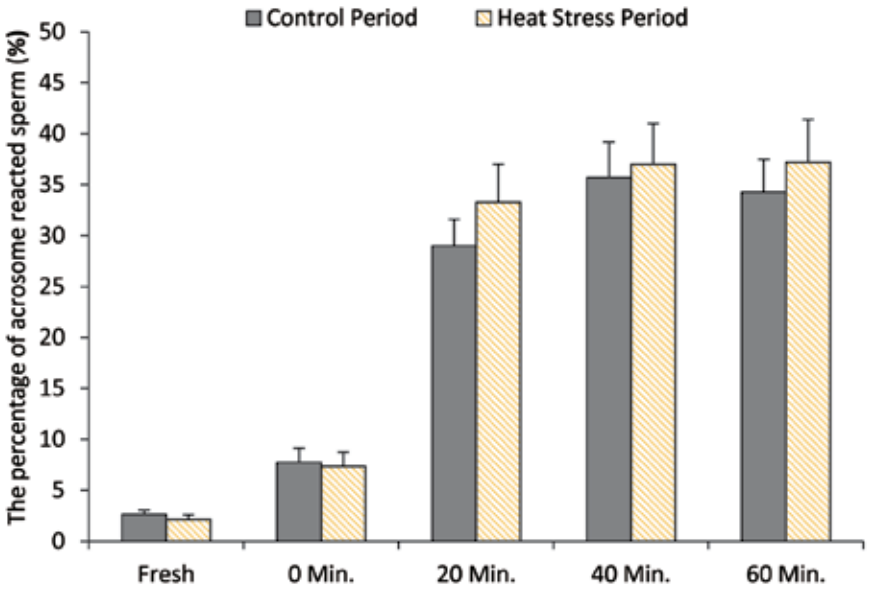

Figure 4. Effect of heat stress on acrosome reacted sperm rates in fresh semen and at 0 min (after 1 h incubation), 20, 40 and 60 min after LPC treatment, respectively ($P > 0.05$).

DISCUSSION

The testicular temperature is lower than body temperature in most livestock and this is necessary for successful spermatogenesis (Banks et al., 2005). The optimum scrotal temperature for spermatogenesis is maintained by specialized tissues such as muscles, sweat glands and pampiniform plexus (Setchell, 2006; Setchell and Breed, 2006; Blazquez et al., 1988). However, high environmental temperature can cause an increase in scrotal temperature, production of reactive oxygen species (ROS) and trigger oxidative stress in the testes (Nichi et al., 2006). Several antioxidants and various types of heat shock proteins have important roles in preventing the detrimental effects of heat stress on sperm (Rockett et al., 2001; Nichi et al., 2006; Hamilton et al., 2016). Despite such defense mechanisms, heat stress may adversely affect sperm quality in most mammals.

In the present study, semen volume was similar in heat stress and control periods. However, sperm concentration was lower during heat stress period compared to the control period ($P < 0.05$) probably as a result of heat stress induced spermatogenic failure (Jannes et al., 1998) and induction of apoptosis in germ cells especially in spermatocytes (Rockett et al., 2001). Heat stress also decreased sperm motility, viability and morphology ($P < 0.05$). Previously it has been reported that heat stress can damage seminiferous epithelium (Rockett et al., 2001) and sertoli cells (Hassanpour et al., 2015). Moreover, heat stress affects the epithelium of cauda epididymis, alters ion concentrations, protein composition of epididymal fluid and inhibits the special ability of the cauda to store and prolong the life of sperm cells (Bedford, 1991). Therefore, such unfavorable testicular and epidydimal environment might account for the lower motility, viable and morphologically normal sperm rate during heat stress period (Fig. 3).

Even though there were some studies focused on effect of environmental heat stress on some sperm parameters in livestock, the present study is unique because it investigates effect of environmental heat stress on ability of sperm to undergo acrosome reaction, sperm plasma membrane and DNA integrity in rams. The sperm plasma membrane is particularly rich in polyunsaturated fatty acids and highly vulnerable against lipid peroxidation, which is triggered by over production of ROS and oxidative stress (Vernet et al., 2004). Additionally, compared to other species ram sperm plasma membrane is more susceptible to lipid peroxidation due to its relatively high polyunsaturated/saturated fatty acid content and low proportions of cholesterol/phospholipids content in the plasma membrane (Darin-Bennett and White, 1977). The present study indicated that heat stress damaged plasma membrane integrity of ram sperm is in agreement with the previous results of Hamilton et al. (2016). The higher sperm plasma membrane disorders in heat stress group might be resulted from lipid peroxidation that induced by over production of ROS during heat

stress period.

Although Hamilton et al. (2016) indicated that insulation of testes increased acrosome damaged sperm rate in rams, Maya-Soriano et al. (2015) reported that environmental heat stress did not altered acrosome damaged sperm rate in rabbits. Maya-Soriano et al. (2015) explained the similar acrosome damaged sperm rates between environmental heat stress and control group as a result of adaptation of rabbits to the extended heat stress conditions. Previous studies also reported that immersion of the testes in 40 to 42°C water bath increased the DNA damaged sperm rate compared to control in mice (Banks et al., 2005; Sailer et al.,1997). In the present study, DNA damaged and acrosome reacted sperm rates were found similar during the heat stress and control periods. The similarity of DNA damaged and acrosome reacted sperm rates in heat stress and control periods might be related to the adaptation of Kıvırcık rams to the extended heat stress condition. Furthermore, unfavorable effect of heat stress on sperm DNA and acrosomal integrity might be limited by specialized tissues to maintain optimal scrotal temperature such as musculus cremaster, scrotal sweat glands in present experimental conditions. In our study, musculus cremaster and scrotal sweat glands were more effective to maintain optimal scrotal temperature compared to previous studies utilizing artificial local heating of testes. Artificially local heating of testis with insulation material or hot water completely block functions of these defense systems. Additionally, ram sperm nuclei includes only Protamine I (Balhorn, 1982) which is maximally cross linked by disulphide bridges, and more stable than mouse sperm nuclei including Protamine I and II (Motoishi et al., 1996). On the other hand, when the lower sperm concentration during heat stress period considered as evidence in present study, similar DNA damaged sperm rates during heat stress and control period might be related to the elimination of DNA damaged germ cells (spermatocytes and early spermatids) by apoptotic defense mechanisms during heat stress period.

CONCLUSION

Environmental heat stress unfavorably influenced sperm concentration, motility, viability, morphology and plasma membrane integrity in *K*ıvırcık rams. However, semen volume, sperm DNA integrity and the ability of sperm to undergo acrosome reaction were not altered under the present environmental heat stress conditions.

CONFLICT OF INTEREST

None of the authors has any conflict of interest to declare. All the authors are aware of submission and agreed to be listed as co-author.

ACKNOWLEDGEMENT

The present manuscript was derived from PhD thesis of Niyazi Küçük. The authors are thankful to BAP (Project number VTF-13011), Aydın Adnan Menderes University for financial support to carry out the research.

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