Impacts of *in vitro* thermal stress on ovine epididymal spermatozoa and the protective effect of β-mercaptoethanol as an antioxidant

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Abstract

Most aspects of reproductive function including spermatogenesis, oocyte growth and maturation, early embryonic development, fetal and placental growth, and lactation can be affected by thermal stress. Furthermore, it has been shown that oxidative stress involves in the pathology of thermal stress. Therefore, the aim of this study was to investigate the impacts of thermal stress on the ovine mature epididymal spermatozoa extracted from testes of slaughtered rams in the presence or absence of an antioxidant. Epididymal spermatozoa were incubated at scrotal (32.00 ℃), normal body (39.00 ℃), and hyperthermic temperatures (41.00 ℃) for 4 hr in the presence or absence of 1 mmol L^{-1} β-mercaptoethanol. The results demonstrated the high sensitivity of ram epididymal spermatozoa to the hyperthermic temperature at *in vitro* conditions. In comparison with scrotal temperature, quality parameters of spermatozoa were negatively affected by increase in temperature, as such in the spermatozoa incubated at hyperthermic temperature significant decrease was observed in the viability, DNA integrity and in the majority of motility parameters. Moreover, concentration of lipid peroxidation by-products, thiobarbituric acid reactive substances, were significantly increased. The findings showed that using antioxidant during incubation period had significant protective effect on the viability and motility of incubated spermatozoa not only at the hyperthermic temperature, but also at the scrotal and normal body temperatures. In conclusion the ovine epididymal spermatozoa were sensitive to *in vitro* thermal stress and it seems that this sensitivity was partly related to the oxidative stress.

Introduction

Thermal stress impacts many biological functions of ovine such as feed intake efficiency and utilization, disturbances in water, protein, energy and mineral balances, enzymatic reactions, hormonal secretions and blood metabolites. In the other words, the productive and reproductive performance of the animal are compromised by this type of stress especially in the warmer parts of the world and in the regions that are affected with global climate changes.[1] Most aspects of reproductive function including spermatogenesis, oocyte development, oocyte maturation, early embryonic development, fetal and placental growth, and lactation can be affected by thermal stress.[2] It has been shown that increased ambient temperature leads to considerable elevated scrotal skin and testicular temperatures,[3] which in turn leads to decreased semen quality parameters such as sperm concentration, motility, viability, morphology, and acrosome integrity.[2,4] Consistently, increased testicular temperature in rams led to decreased sperm count, increased sperm abnormalities and changed seminal plasma proteome.[5] In addition, inseminating females with the semen of heat stressed males led to elevated fertilization failure and increased embryonic mortality.[6,7]

Nowadays, experimental evidences support the idea that oxidative stress is involved in the pathology of thermal stress.[8-10] It has been shown that thermal stress through different pathways results in oxidative stress: Heat stress increases H_2O_2 production,[11] hydroxyl radical

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formation,[12] and mitochondrial superoxide anion levels.[13] It was also shown that thermal stress decreased cytoplasmic and mitochondrial superoxide dismutase protein and enzyme activity, leading to the increased reactive oxygen species (ROS) generation.[14,15] Further-more, thermal stress results in a substantial decrease in glutathione (GSH) levels.[16] Although ROS have important roles in the sperm physiological processes such as capacitation, hyperactivation, and sperm-oocyte fusion,[17] the excessive amount of ROS damages spermatozoa.[18] These oxygen species cause lipid peroxidation, protein oxidation, DNA base modifications, and DNA strand breaks.[9]

Post-mortem recovery of epididymal spermatozoa from the epididymis of a dead animal could be a useful option in the situations that a genetically valuable male dies accidentally or must be culled from the flock.[19] Sperm collection must be performed in a suitable extender, and collected spermatozoa can be used either immediately in artificial insemination, *in vitro* embryo production programs or stored for later utilization as liquid or cryopreserved.[20] Epididymal spermatozoa collections have a sufficient number of viable spermatozoa that can be used to fertilize oocytes, and the resulting embryos are able to develop into the healthy live neonates.[21] Therefore, investigating factors affecting *in vitro* function of this type of spermatozoa in different conditions may provide useful information.

Considering thermal stress impacts on the male fertility and sperm physiology, as well as importance of epididymal spermatozoa as an option for genetic preservation using an *in vitro* model, we performed this study to investigate the effects of thermal stress on the epididymal spermatozoa of rams. Besides, due to the increasing evidences indicating the role of excessive ROS production in the thermal stress pathology, the influence of β-marcaptoethanol as a well-known thiol antioxidant was also evaluated. Evaluated endpoints included motility and kinematic parameters, viability or functional membrane integrity, DNA integrity, and thiobarbituric acid reactive substances (TBARS) assay as an indicator of lipid peroxidation.

Materials and Methods

All salts, acridine orange, thiobarbituric acid and β-mercaptoethanol were obtained from Merck (Darmstadt, Germany). Penicillin, streptomycin and 4-(2-hydroxy-ethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Sigma Chemicals Co. (St. Louis, USA). All experiments were performed during April and May in Shahrekord, Charmahal-va-Bakhtiary province, Iran (32°19′N, 50°51′E).

Experimental design. This experiment included six experimental groups: Three groups without antioxidant and three groups with antioxidant (AO). In order to perform one replicate of the experiment, spermatozoa obtained from three testes were pooled and the pooled sample was kept at room temperature for 30 min. Then the motility of the sample was analyzed using computer-assisted sperm analysis (CASA, Hooshmand Fanavar, Tehran, Iran) system. Where, the total and progressive motility of the sample were over 50.00% and 35.00%, respectively, then the sample was used for performing the experiment. At first, samples were prepared for assessing the DNA integrity, viability, and lipid peroxidation of the fresh samples. Then aliquots of 2.00×10^7 sperm mL^{-1} in sperm medium were made in order to incubate at three different temperatures: Scrotal temperature (ST; 32.00 ℃), normal body temperature (NT; 39.00 ℃) and heat stressed body temperature (HT; 41.00 ℃). Along with each antioxidant- free sperm aliquot, an antioxidant-containing aliquot (1.00 mmol L^{-1} β-mercaptoethanol) was incubated at the intended temperature. The experimental groups were as follows: ST, ST-AO, NT, NT-AO, HT, and HT-AO. The incubation process was performed in three independent incubators. At the end of incubation period, in each incubated sperm aliquot the motility, viability, DNA damage, and the level of TBARS were evaluated. This experiment was performed in 10 replicates.

Sperm recovery. Testes of sexually mature rams were removed immediately after the slaughter from carcasses and transported to the laboratory at 4.00 ℃. Sperm recovery was performed immediately in laboratory at almost 2 hr post-slaughter. After removing tunica albuginea covering the tail of the epididymis, an incision was made on the ventral surface of the epididymis using a scalpel blade and the secretions were collected and resuspended in 1.00 mL sperm medium (114 mmol L^{-1} NaCl, 3.20 mmol L^{-1} KCl, 5.00 mmol L^{-1} NaHCO$_3$, 0.40 mmol L^{-1} NaH$_2$PO$_4$·H$_2$O, 2.80 mmol L^{-1} sodium lactate, 0.50 mmol L^{-1} MgCl$_2$·6H$_2$O, 5.00 mmol L^{-1} sodium bicarbonate, 20.00 mmol L^{-1} HEPES, 1.50 mmol L^{-1} glucose, 100 IU mL^{-1} penicillin-G, and 100 μg mL^{-1} streptomycin).

Motility assessment. Assessment of motility para-meters was carried out using CASA system. Five microliters of sperm suspension were loaded on a pre-warmed Spermmeter™ semen analysis chamber (10.00 μm depth; Sperm Processor Pvt. Ltd., Aurangabad, India) and then evaluated. For each sample, six vision fields were analyzed with 30 frames per sec. Magnifying power was 4× for microscope objective lens. The motility descriptors obtained after CASA analyses were total motility (TM; %), progressive motility (PM; %), curvilinear velocity (VCL; μm sec^{-1}), linear velocity (VSL; μm sec^{-1}), mean velocity (VAP; μm sec^{-1}), mean angular displacement (MAD; degrees), mean amplitude of lateral head displacement (ALH; μm), frequency of head displacement (BCF; Hz), linearity coefficient (LIN; %); wobble coefficient (WOB; %), and straightness coefficient (STR; %).

Viability assessment. Viability was assessed using hypo-osmotic swelling test according to WHO laboratory manual for the examination and processing of human semen. In brief, 30.00 μL of sperm sample was diluted in 300 μL of swelling solution (25.00 moml L^{-1} sodium citrate dehydrate and 75.00 mmol L^{-1} D-fructose) and was incubated at 37.00 ℃ for 30 min. After the incubation, 10.00 μL of suspension was placed on a grease-free microscope slide and covered by a coverslip and examined by a phase-contrast microscope with 40× objective. Two slides per each sample were prepared and 200 sperm were counted on each slide. Percent of live spermatozoa was calculated by the division of spermatozoa with swollen tail (live) to total counted sperm.[22]

DNA integrity assessment. Sperm DNA integrity assessment was performed by acridine orange staining assay. For preparing staining solution, 10.00 mL of 0.10 mg mL^{-1} aqueous solution of acridine orange was mixed with 40 mL of 0.10 mol L^{-1} citric acid and 2.50 mL of 0.30 mol L^{-1} disodium hydrogen phosphate. A thin smear of sperm sample was prepared on grease free slides and air dried for at least 15 min and then fixed in Carnoy's fixative (3:1, methanol-glacial acetic acid) at room temperature for 2 hr. After rinsing with distilled water, the slides were covered by the staining solution and kept for 10 min. After rinsing and mounting coverslip, the slides were observed under an epifluorescence microscope using 490 nm excitation filter and 530 nm barrier filter and a total number of 200 sperms was counted on each slide. Acridine orange fluoresces green when it intercalates into native DNA (double-stranded and normal) as a monomer and red when it binds to denatured (single-stranded DNA) as an aggregate.[23] Green sperms indicated sperms with intact DNA (non-damaged) and red/yellow sperm indicated sperm with damaged DNA.

TBARS assay. Malondialdehyde (MDA), a product of lipid peroxidation, is a major substance that reacts with thiobarbituric acid. Using the TBARS assay, MDA was measured according to Yagi.[24] Briefly, sperm suspension was sonicated for cell lysate. 600 μL of 10.00% (v/v) trichloroacetic acid solution were added to the sonicated sperm suspension (2:1) in order to precipitate proteins and cellular debris. Samples were centrifuged (16,000 *g*, for 10 min) and the supernatant recovered. Then an equal volume of thiobarbituric acid 1.00% solution (in NaOH 0.05 mol L^{-1}) was added to the supernatant and the samples were incubated in a boiling water bath for 10 min. After cooling samples on ice, absorbance was measured at 532 nm by a spectrophotometer (Corning Inc., New York, USA). The TBARS concentration was calculated from standard curve previously prepared and expressed as μM mg^{-1} of the total proteins of the sperm suspension. Total proteins of the sperm samples were measured by Bradford method.[24]

Statistical analysis. The data were analyzed using the Statistical Analysis System software (version 9.1; SAS Institute, Cary, USA). The factorial ANOVA model were used to determine the statistical differences among the main effects of the incubation temperatures, antioxidant presence and their interactions (temperature × antioxidant). If a non-significant interaction was detected, the main effects (temperature and antioxidant) were interpreted. The comparison of means among the experimental groups and between experimental groups and fresh group was performed using one-way ANOVA followed by Tukey's test. Graphs were created using GraphPad Prism (version 5.0; GraphPad Software Inc., San Diego, USA). The data expressed as mean ± SEM and differences at a level of *p* < 0.05 were considered as statistically significant.

Results

The data of the main effects of incubation temperatures and antioxidant along with model analysis results are presented in Tables 1, 2 and 3. In these Tables the mean values of temperature effects, irrespective to the presence or absence of antioxidant, and the mean values of presence or absence of antioxidant, irrespective to the incubation temperature, are presented. The results of fresh group and different experimental groups are presented in Figure 1. The results showed that both the incubation temperatures and antioxidant affected the majority of the motioned parameters of spermatozoa (*p* < 0.05). However, the interaction of main effects was not significant in this respect (Tables 1 and 2). Irrespective to the incubation temperature, in comparison with initial fresh group in antioxidant-free groups all the motility parameters of sperm samples were negatively affected by the incubation process (Fig. 1). Regarding incubation temperature, incubation at 41.00 ℃ had greater detrimental effect on the motility parameters than other temperatures (*p* < 0.05), (Tables 1 and 2). Presence of antioxidant during incubation period, irrespective to the incubation temperatures, led to the neutralization of the detrimental effects of incubation on the motility so that all values in antioxidant-containing groups were significantly higher than their antioxidant-free counterparts (*p* < 0.05), (Fig. 1, Tables 1 and 2). Presence of antioxidant during incubation period maintained the motility parameters at the level of fresh group (Fig. 1).

The main effect of incubation temperature and presence of antioxidant on the viability of the incubated spermatozoa was significant (*p* < 0.05), however, the interaction of main factors was not (Table 3). Viability rate of spermatozoa under the influence of incubation process and increasing incubation temperature was decreased, as such all groups, except for ST-AO, showed significantly lower viability rate than fresh group (*p* < 0.05), (Fig. 1).

Regarding incubation temperature, incubation at 41.00 °C showed greater detrimental effect on the viability than other temperatures (Table 3). Presence of antioxidant during incubation period, irrespective to the incubation temperatures, led to the neutralization of the detrimental effects of incubation on the viability so that the viability in the antioxidant-containing groups was significantly higher than their antioxidant-free counterparts ($p < 0.05$), (Fig. 1 and Table 3). Presence of antioxidant during the period of incubation at each temperature led to the significantly better preservation of viability than its antioxidant-free counterpart ($p < 0.05$), (Fig. 1). The incubation temperature had a significant effect on the DNA integrity of incubated spermatozoa ($p < 0.05$), however, the presence of anti-oxidant during incubation period and also the interaction of antioxidant and incubation temperature had no effect (Table 3). Incubation of spermatozoa at 41.00 °C led to a significant increase of spermatozoa with damaged DNA compared to the fresh group, ST and NT groups ($p < 0.05$), (Fig. 1 and Table 3). Presence of antioxidant in the incubation medium had no significant effect on the rate of DNA damage ($p < 0.05$), (Table 3).

Table 1. The main effects of incubation temperature, presence or absence of antioxidant during incubation period, and the interaction of the main effects on some motility parameters of ovine epididymal spermatozoa.

Parameters		TM (%)	PM (%)	VCL (μm sec^{-1})	VSL (μm sec^{-1})	VAP (μm sec^{-1})
	32.00	46.30 ± 2.77^a	36.00 ± 2.75^a	42.10 ± 2.65^a	20.00 ± 2.09^a	25.20 ± 2.18^a
Temperature (°C)	39.00	44.10 ± 2.26^{ab}	32.70 ± 2.10^{ab}	38.40 ± 1.98^{ab}	14.90 ± 1.28^b	20.30 ± 1.33^{ab}
	41.00	37.30 ± 2.23^b	27.30 ± 2.44^b	32.60 ± 1.73^b	12.10 ± 1.23^b	16.80 ± 1.28^b
Antioxidant	-	34.60 ± 2.35*	24.80 ± 2.21*	30.50 ± 1.92*	11.70 ± 1.41*	16.30 ± 1.48*
	+	50.50 ± 2.06	39.20 ± 2.36	44.90 ± 1.88	19.60 ± 1.57	25.30 ± 1.57
		***p*-value**				
Temperature		0.0061	0.0073	0.0010	0.0002	0.0002
Antioxidant		< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Temperature × Antioxidant		0.6849	0.7129	0.6949	0.8698	0.8671

TM: Total motility, PM: Progressive motility, VCL: Curvilinear velocity, VSL: Linear velocity, VAP: Mean velocity.
^{ab} Different letters in each column indicate significant differences between temperature main effects ($p < 0.05$).
* Asterisk in each column indicates significant difference between presence or absence of antioxidant main effects ($p < 0.05$).

Table 2. The main effects of incubation temperature, presence or absence of antioxidant during incubation period, and the interaction of the main effects on some other motility parameters of ovine epididymal spermatozoa.

Parameters		MAD (degrees)	ALH (μm)	BCF (Hz)	LIN (%)	WOB (%)	STR (%)
	32.00	15.70 ± 1.36	2.10 ± 0.08^a	4.10 ± 0.50^a	31.70 ± 1.61^a	46.80 ± 1.30^a	50.80 ± 1.75^a
Temperature (°C)	39.00	16.20 ± 1.49	2.10 ± 0.07^{ab}	3.40 ± 0.41^{ab}	27.80 ± 1.10^{ab}	43.80 ± 0.71^{ab}	48.30 ± 1.33^{ab}
	41.00	13.50 ± 1.01	1.80 ± 0.06^b	2.70 ± 0.33^b	25.90 ± 1.47^b	40.70 ± 1.32^b	44.80 ± 1.81^b
Antioxidant	-	10.60 ± 1.05*	1.80 ± 0.06*	2.10 ± 0.33*	25.40 ± 1.41*	41.10 ± 1.22*	43.40 ± 1.55*
	+	19.70 ± 1.13	2.30 ± 0.06	4.70 ± 0.44	31.50 ± 1.29	46.40 ± 0.92	52.60 ± 1.39
		***p*-value**					
Temperature		0.1528	0.0015	0.0127	0.0137	0.0169	0.0632
Antioxidant		< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Temperature × Antioxidant		0.6217	0.4914	0.8299	0.7449	0.6076	0.8730

MAD: Mean angular displacement, ALH: Mean amplitude of lateral head displacement, BCF: Frequency of head displacement, LIN: Linearity coefficient, WOB: Wobble coefficient, and STR: Straightness coefficient.
^{ab} Different letters in each column indicate significant differences between temperature main effects ($p < 0.05$).
* Asterisk in each column indicates significant difference between presence or absence of antioxidant main effects ($p < 0.05$).

Table 3. The main effects of incubation temperature, presence or absence of antioxidant during incubation period, and the interaction of the main effects on the viability, DNA damage and lipid peroxidation of ovine epididymal spermatozoa.

Parameters		Viability (%)	DNA damage (%)	TBARS (μM mg^{-1} of total proteins)
	32.00	76.20 ± 1.27^a	18.20 ± 0.77^a	0.60 ± 0.04^a
Temperature (°C)	39.00	66.70 ± 2.02^b	20.30 ± 1.44^a	0.67 ± 0.04^{ab}
	41.00	55.70 ± 1.44^c	27.80 ± 0.53^b	0.73 ± 0.03^a
Antioxidant	-	62.40 ± 1.70*	22.70 ± 0.86	0.70 ± 0.03
	+	70.00 ± 1.41	21.50 ± 0.53	0.63 ± 0.03
		***p*-value**		
Temperature		< 0.0001	< 0.0001	0.0318
Antioxidant		< 0.0001	0.3038	0.0393
Temperature × Antioxidant		0.5356	0.3234	0.6279

TBARS: Thiobarbituric acid reactive substances.
^{abc} Different letters in each column indicate significant differences between temperature main effects ($p < 0.05$).
* Asterisk in each column indicates significant difference between presence and absence of antioxidant main effects ($p < 0.05$).
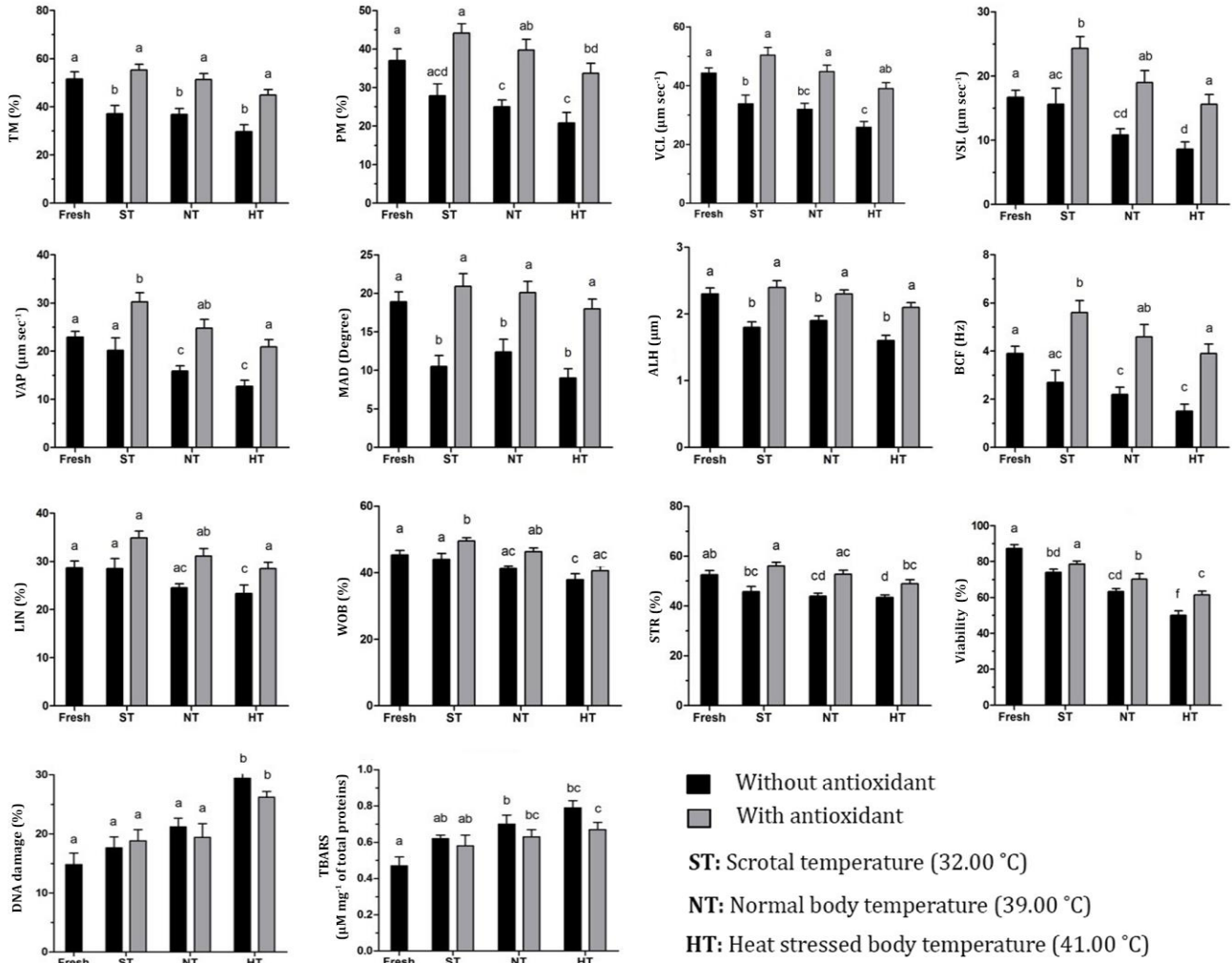
Fig 1. Effects of incubation temperatures and the presence of antioxidant on the evaluated parameters of ovine epididymal spermatozoa. TM: Total motility; PM: Progressive motility; VCL: Curvilinear velocity; VSL: Linear velocity; VAP: Mean velocity; MAD: Mean angular displacement; ALH: Mean amplitude of lateral head displacement; BCF: Frequency of head displacement; LIN: Linearity coefficient; WOB: Wobble coefficient; STR: Straightness coefficient, and TBARS: Thiobarbituric acid reactive substances.
[a-f] Different letters indicate significant differences between experimental groups ($p < 0.05$).

The results showed that both the incubation temperature and antioxidant significantly affected the concentration of TBARS ($p < 0.05$). However, the interaction of main effects was not significant in this respect ($p < 0.05$), (Table 3). Increasing temperature resulted in the increased concentration of TBARS in culture medium so that concentration of TBARS in NT and HT groups was significantly higher than that of the Fresh group ($p < 0.05$) and also there was significant difference between ST and HT groups ($p < 0.05$), (Fig. 1). Although antioxidant had a decreasing effect on the levels of TBARS, this decrease was not significant when the antioxidant-free and antioxidant-containing counterparts were compared ($p < 0.05$), (Fig. 1 and Table 3). However, the concentration of TBARS in HT-AO group statistically had reached to the same level as ST and ST-AO groups (Fig. 1).

Discussion

In the present study we investigated the impacts of thermal stress on the ovine epididymal spermatozoa in the presence or absence of an antioxidant. The results demonstrated high sensitivity of ram epididymal spermatozoa to hyperthermic temperature at *in vitro* conditions. Although we observed that the quality parameters of spermatozoa were negatively affected by incubation even at scrotal and normal body temperatures, the worst effects were seen at hyperthermic temperature. In comparison with ST, quality parameters of spermatozoa were negatively affected by the increasing temperature in the spermatozoa incubated at HT and significant decrease was observed in the viability, DNA integrity and in the majority of motility parameters, while concentration of lipid

peroxidation by-products, TBARS, was significantly increased. Meanwhile, our findings showed that using anti-oxidant during incubation period had significant protective effect on the viability and motility of the incubated spermatozoa not only at the hyperthermic temperature, but also at the scrotal and normal body temperatures.

Our results demonstrated that in addition to hyper-thermic temperature, even at scrotal and normal body temperatures quality parameters of incubated epididymal spermatozoa were affected. This condition was improved by the presence of antioxidant during the incubation period. Thus, it seems that adverse effect of the incubation on spermatozoa might have an oxidative nature. By progressive decrease in the cytoplasm of spermatozoa during spermiogenesis, the antioxidant potential of sperm cells concomitantly decreases and reaches to its minimal state in the mature epididymal spermatozoa.[25] The environment of the epididymis has a variety of antioxidant mechanisms that protects vulnerable sperm cells against oxidative damage.[26] Spermatozoa in this milieu also are immotile and have low metabolic activity.[27] Upon separating spermatozoa from the epididymal environment and diluting in culture media, they gradually become active and their metabolic rate increases. Consequently, energy substrates of media will be depleted and also harmful byproducts of metabolism such as ROS will be accumulated. In addition, the sperm cell, especially the immature and defective one, has a significant ability to generate ROS.[28,29] Collectively, incubating a cell with low anti-oxidant potential and also with a high capability to generate ROS in an antioxidant-free environment which encourages high rate of metabolic activity and might lead to the occurrence of oxidative stress and its destructive consequences.

Similarly, it has been shown that incubation of bovine spermatozoa even at normothermic temperature for a short period of time compromised their quality parameters.[30-33] In ovine, scrotal hyperthermia leads to reduced sperm count, lower proportion of motile sperm, decreased integrity of sperm plasma membrane and greater proportion of spermatozoa with abnormal morphology.[34,35] Although spermatocytes and round spermatids are the most sensitive stages of spermatogenetic process,[4,36] mature spermatozoa in the caudal portion of epididymis also are impacted by thermal stress to some extent.[4,6] Despite the extensive work done on the influences of environmental and scrotal hyperthermia on the testis physiology and the development of spermatozoa, studies on the direct effects of thermal stress on mature spermatozoa function, both ejaculated and epididymal, are less frequent. At *in vitro* models quality parameters of spermatozoa such as motility, viability, membrane integrity, DNA integrity, acrosomal intactness, and fertilizing capability have been negatively affected by hyperthermic treatment and this negative effect was much greater than that of normo-thermic treatments.[30,32,37]

Consistently, in our study the most intense decrease in the viability, motility, and DNA integrity of spermatozoa and also the highest level of TBARS were observed at hyperthermic temperature. Similar to ST-AO and NT-AO groups, antioxidant had some protective effects at this temperature that were lesser than those seen in ST-AO and NT-AO groups. As stated above, pathophysiology of heat stress involves overproduction of various oxidant agents through different pathways. It has been shown that oxidative stress disrupts both ATP production by oxidative phosphorylation process, and ATP utilization by the sperm motor machinery, which both lead to motility cessation.[38] Also, excessive amounts of ROS cause sperm plasma membrane damage by triggering the lipid peroxidation of membrane lipids.[18] Polyunsaturated fatty acids of cell membranes are susceptible to peroxidation by ROS that has numerous physiological consequences.[39] Ram, bull and boar spermatozoa when compared to other species have an excessively high proportion of polyunsaturated/ saturated fatty acids and low proportion of cholesterol/ phospholipid in the plasma membrane.[40,41] As a result, the sperm membrane of these species is more sensitive to lipid peroxidation by ROS. The continued oxidation of fatty acid side-chains and released PUFAs eventually leads to the loss of membrane integrity by the alteration of its fluidity which finally triggers the inactivation of membrane-bound proteins.[42] As a result of membrane lipid peroxidation, the irreversible loss of motility in ram, bull, stallion, rat, boar, rabbit, rooster and mouse spermatozoa has been observed.[40] In addition, lipid peroxidation produces toxic derivatives such as MDA and 4-hydroxy-nonenal (4-HNE). The MDA appears to be the most mutagenic product of lipid peroxidation, whereas 4-HNE is the most cytotoxic.[43] It has been observed that there is a very strong negative correlation between MDA levels and sperm motility, viability and acrosomal integrity.[44]

In our study DNA damage or the susceptibility of chromatin to acid-denaturation was significantly increased after incubating spermatozoa at hyperthermic temperature in comparison to the ST and NT groups. Although the antioxidant had beneficial effects on the motility and viability at this temperature, it failed to prevent DNA damage. Damage to the chromatin of spermatozoa due to *in vitro* heat stress[32] or even by *in vitro* incubation at normothermic temperatures[45,46] has been previously reported, and due to ROS overproduction in these situations, damage to the DNA of spermatozoa possibly was associated to oxidative stress. It has been shown that *in vitro* exposure of mature sperms to ROS can induce significant DNA damage[47,48] and this damage can be triggered by direct action of ROS on the sperm DNA or indirectly through the activation of sperm caspases and endonucleases.[49] In this study, ineffectiveness of anti-oxidant on preventing DNA damage might be related to the nature of acridine orange test and also to the thiol

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antioxidant that used in this experiment. Acridine orange staining process has an acid denaturation step in which mild acid treatment denatures the DNA with single stranded or double stranded breaks. Kosower *et al.* showed that by treating sperm nuclei with a thiol agent (dithiothreitol) before acridine orange staining, percent of nuclei with red signal was increased. They concluded that sperm DNA was vulnerable to acid denaturation when their DNA-associated protamines were poor in disulfide bonds.[50] The β-mercaptoethanol is a reducing agent that can reduce the disulfide bonds of DNA-linked protamines, and because of this property is a component of some sperm DNA extraction kits. It could be inferred that β-mercaptoethanol was successful in neutralizing excessive ROS produced during incubation, however, through reducing disulfide bonds of sperm nuclei, it introduced an artifact in the acridine orange assay which either was a kind of DNA damage or was misinterpreted as DNA damage.

In conclusion, ovine epididymal spermatozoa were sensitive to *in vitro* incubation and it seems that this sensitivity was related to oxidative stress. In addition, incubating ram spermatozoa at higher temperatures exerted greater negative effects on the sperm quality. Although β-mercaptoethanol had neutralized some detrimental effects of *in vitro* incubation and hyper-thermic treatments on the ovine epididymal spermatozoa and this antioxidant had negative effects on DNA integrity; therefore, β-mercapto-ethanol must be used by caution for ameliorating the effects of oxidative stress on spermatozoa.

Acknowledgments

Conflict of interest

The authors declare no potential conflict of interest.

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