ORIGINAL ARTICLE

Veterinary Research Forum. 2022; 13 (1) 61 – 70
doi: 10.30466/vrf.2020.124845.2927
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

Evaluation of dietary betaine on post-thawed semen quality in mature bulls during summer heat stress

Reza Ijab[1], Esmail Ayen[1]*, Amir Khaki[2], Ali Soleimanzadeh[1]

[1] Department of Theriogenology and Poultry Disease, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran; [2] Department of Clinical Sciences, Faculty of Veterinary Medicine, Amol University of Special Modern Technologies, Amol, Iran.

Article Info

Article history:

Received: 28 June 2020
Accepted: 28 November 2020
Available online: 15 March 2022

Keywords:

Betaine
Bulls
Heat stress
Semen quality

Abstract

Heat stress (HS) has caused relative hypoxia, oxidative stress and high level of homocysteine, which contributes significantly to fertility failures in bulls. The aim of present study was to evaluate the role of dietary betaine (BET) in improving dual purpose Simmental (Fleckvieh) post-thawed semen quality especially during the hottest summer days. A total number of 16 mature bulls were randomly assigned to three equal groups including: 1) Control condition (without betaine), 2) BET1: 57.00 mg of betaine kg^{-1} per day and 3) BET2: 114 mg of betaine kg^{-1} per day, through daily intakes for 90 days in summer. Plasma levels of homocysteine, seminal plasma antioxidants levels and sperm parameters such as DNA fragmentation, chromatin integrity, motility, viability, morphology and membrane integrity were evaluated. Under maximal HS, serum homocysteine concentrations were reached 16.67 ± 0.09 μmol L^{-1}. Dietary betaine supplementation influenced DNA fragmentation of sperm and was higher in the control group compared to BET2 group. There were significant decreases in seminal plasma superoxide dismutase (SOD), glutathione peroxidase (GPx) activity and sperm viability and motility in bulls treated with betaine. The activity of GPx and SOD in the control group was increased up to 0.08 ± 0.00 U mg^{-1} protein and 0.52 ± 0.01 U mg^{-1} protein in seminal plasma. There were no significant differences between groups in the percentage of swollen spermatozoa, membrane integrity, sperm morphology, abnormal head morphology and percentage of spermatozoa stained with aniline blue. In conclusion, BET supplements improved semen parameters in sperm motility, sperm viability and influenced DNA fragmentation during HS with reduction in serum homocysteine concentrations.

Introduction

When bulls are exposed to higher ambient temperature, heat stress (HS) occurs. However, humidity and air flow exceed the ability of the body to remove the extra heat.[1] The *Bos Taurus* breed (Simmental breed) is less heat-tolerant than *Bos Indicus* cattle during the hot summer months in tropical areas because thermolysis mechanisms are more slow.[2] Total duration of spermatogenesis and epididymal maturation in bulls was estimated to take about 65 days. However, these adverse effects of HS could appear within two weeks especially as a decreased sperm motility.[3]

Heat stress may prompt reduced bull fertility such as increase in abnormal sperm and reduction in sperm concentration. Increased bovine testicular temperature beyond 33.00 - 34.44 ˚C causes increase in metabolic rates and oxygen requirements.[4] Relative hypoxia theoretically adversely affects spermatogenesis and sperm function by reactive oxygen species (ROS).[5] For instance, the ROS leads to damage spermatozoa through lipid peroxidation of the plasma membrane and high amount of polyunsaturated fatty acids (PUFA). This process has been shown to be present in sperm membranes, thereby, impeding membrane fluidity and flexibility.[6] The HS has been related to decreased sperm motility, concentration, and viability in mice,[7] and bull,[8] which can disrupt spermatogenesis, causing germ cell death and subfertility.

Betaine (BET; trimethylglycine), derived from choline, is a zwitterion that may help relieve HS in livestock species

***Correspondence:**

Esmail Ayen. DVM, PhD
Department of Theriogenology and Poultry Disease, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran
E-mail: e.ayen@urmia.ac.ir

and elevate their reproductive performance. The principle role for BET in the body is as a methyl donor and an osmoprotectant. BET donates one of its methyl groups to convert homocysteine (Hcy) into L-methionine.[9] High level of Hcy in seminal plasma and blood is negatively correlated with the sperm count, motility and DNA sperm fragmentation.[10] Furthermore, higher levels of Hcy level have been increased markedly with oxidative stress or response to heat shock. Although Hcy has been a sulfur containing amino acid for the growth of cells, Hcy toxicity such as protein structural modifications, oxidative stress and neurotoxicity have been associated with elevated levels of Hcy. The Hcy can be metabolized via two alternative pathways, the transsulfuration pathway allows conversion of methionine into cysteine and/or converted back to methionine through remethylation pathway, betaine is involved in this pathway.[11] Numerous studies have been carried out to investigate the potential, nutritional and physiological functions of betaine supplementation on animal performance.[9,12] The osmoregulation-related properties of BET, such as water solubility and dipolar zwitterion properties help maintain the balance of fluid both inside and outside of cells in stress-related conditions like HS condition during summer.

The majority of the existing *in vivo* studies showed dietary BET could improve reproductive and/or productive performance during HS in rabbit bucks,[13] boars,[14] lactating Holstein cows,[15] lactating sows[16] and male mice.[7] The following hypotheses were tested in the present study: Dietary BET supplementation would lead to reduce the effect of HS on post-thawed semen parameters and an improvement of post-thaw semen quality in fertile bulls.

Materials and Methods

Animals and experimental design. The experiment was performed at the Simmental Cattle Breeding Center, Amard-dam Company, Amol, Iran (height above sea level: 47m, 36° 28′ 11″ N, 52° 21′ 3″ E). Semen was collected from 15 mature dual purpose Simmental (Fleckvieh) fertile bulls with the live body weight ranged within 650 - 1,100 kg and aged 3 to 6 years that used as frozen semen producer from June 2019 to September 2019 between 8:00 and 12:00 A.M. The sexual stimulation of bulls was carried out by 10 min standing near the dummy (a restrained bull in the box) with three false jumps. Bulls were maintained under similar conditions of feeding and housing, in which all the animals received the same diet. The animals were fed three times per day based on following Fleckvieh bulls' daily diet formula: Silage 18.00 kg including 8.50% crude protein, 54.50% neutral detergent fiber (NDF), 32.70% acid detergent fiber (ADF), 1.80% fat, 5.70% ash, and 25.00% dry matter), concentrate 9.00 kg (14.68% crude protein, 16.80% NDF, 13.10% ADF, 3.20% fat, 7.40% ash, and 89.20% dry matter), alfalfa 3.00 kg, straw and water *ad libitum*, calcium 0.74%, phosphorus 0.53%, sodium 0.49%, magnesium 0.29%, zinc 375 ppm, manganese 381.44 ppm, cobalt 1.01 ppm, selenium 2.75 ppm plus mineral and vitamin supplements. We attempted to monitor daily weather data including minimum and maximum for ambient temperatures and relative humidity. Rectal temperature (RT) was also measured. Semen samples were categorized into four groups according to the following timings: Early stages of HS, T1 (June) and T2 (July), late stage of HS, T3 (August) and after periods of HS, T4 (September). Temperature-humidity index (THI) was calculated:

$$THI = (1.8 \times T + 32) - [(0.55 - 0.0055 \times RH) \times (1.8 \times T - 26)]$$

where, T is the air temperature in ˚C, and RH is the relative humidity in percent (%).[17] THI thresholds for HS in cattle was reported as follows: Comfort (THI < 68), mild discomfort (68 < THI < 72), discomfort (72 < THI < 75), alert (75 < THI < 79), danger (79 < THI < 84) and emergency (THI > 84).[18] Animals were randomly divided into three groups of five bulls each to receive betaine (96.00% betaine, Vistabet, AB Vista, Marlborough, UK): without BET (control group), 57.00 mg of BET kg^{-1} of BW per day (BET1) and 114 mg BET kg^{-1} of BW per day (BET2).[15] Bulls in all groups were fed on 100 g of premix daily consisting ground corn with different contents of BET for treatment groups and without betaine (placebo) for control group for 90 days. However, measurement variables were followed for 120 days to investigate the effects of HS in control group.

Semen collection. Semen ejaculates were collected twice a week (2 to 3-day intervals) using an artificial vagina (45.00 ˚C) and ejaculate was collected from each bull two times in a day of collection. Semen samples were immersed in the water bath at 32.00 ˚C until semen evaluation. Semen volume and concentration were measured by a sterile graduated glass vial and SDM photometer (Minitube, Tiefenbach, Germany) calibrated for bull sperm cell counting, respectively.

Semen processing, freezing, and thawing. One step dilution method (room temperature semen packaging) was applied for semen freezing through following procedure. In brief, semen samples with motility of at least ≥ 70.00% and normal morphology and viability ≥ 80.00% were used for freezing. Pre-extender dilution was prepared with gentle addition of the extender (Steridyl CSS, Minitube, Germany) to the semen (with ratio of 1:1) and then placed in a water bath at 34.00 ˚C for 10 min. The extender was Steridyl CSS (Minitube). It was a ready to use TRIS (Minitube) base extender which already contained the egg yolk in the concentrate. Only 750 mL of pure water has to be added to 500 mL of the concentrate for preparation. Steridyl (minitube) contained TRIS, citric acid, sugar, buffers, glycerol, purest water,

irradiated sterile egg yolk and antibiotics (tylosin, gentamicin, spectinomycin, lincomycin). At this time the volume of final needed extender was calculated using following formula:

Number of doses = (semen volume × semen concentration × progressive motile sperm × morphologically normal sperm) / (sperm per dose [15.00 × 10^6])

After 10 min, final solution was prepared with adding the pre-extender to final calculated extender volume and left at room temperature (20.00 - 24.00 ˚C) for 15 min. Then, the semens were packed into the 0.50 mL straws (Minitube, Čeľadice, Slovakia) with MPP Uno automated filling and sealing machine (Minitube, Tiefenbach, Germany) and placed on the loading and counting tray. Then, the packed straws were kept at 4.00 ˚C for 3 hr in refrigerator in order to reach in equilibration stage. Finally, equilibrated semens were put in MT freezer device (Minitube) at –120 ˚C for 10 min. Frozen straws were stored in the separate goblets into the canisters of the liquid nitrogen container at –196 ˚C.

Measuring sperm motility using CASA. After two weeks frozen–thawed semen samples were analyzed to compare the sperm motility parameters obtained using the computer assisted sperm motility analysis (CASA, version 6.0; Hoshmand Fanavaran, Tehran, Iran). Parameters such as progressive motility (PM), curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), lateral head displacement (ALH), beat cross frequency (BCF), degrees of deviation (MAD) and linearity (LIN; VSL/VCL) were evaluated. All analyses were performed by light microscope equipped with warm plate and maintaining samples at 37.00 ˚C and a chamber (depth of 10 µm, surface graticule of 100 × 0.01 mm^2) to avoid reduction in sperm motility during the analysis.

Assessment of membrane integrity. The hypo-osmotic swelling test (HOST) predicts sperm membrane function. The HOST was carried out following the technique used by Revell and Mrode.[19] Percentages of sperm reacted in a HOST and the sperm with swollen or coiled tails were determined for all bulls.

Sperm viability and morphology assay. Eosin-nigrosin (Minitube) staining was used for viability and morphological abnormality assessment by examination of 200 sperm per sample under 400 x magnification.[20]

Measurement of antioxidants in seminal plasma. Superoxide dismutase (SOD) activity in seminal plasma was determined using commercial kit (Randox, Crumlin, UK) based on the manufacture's manual. One unit of SOD was defined as the amount of enzyme activity required for 50.00% inhibition of 2-[4-iodopheny]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride (INT) reduction. Eventually, results were expressed as U mg^{-1} protein in seminal plasma.[21] Glutathione peroxidase (GPx) was measured using commercial kit (Randox Laboratories Ltd.) according to Paglia and Valentine.[10] The values of enzyme were expressed as specific activity (U mg^{-1} protein in seminal plasma) and total activity (mU per total seminal plasma).

Total Hcy measurement. Serum Hcy was measured using enzyme-linked immunosorbent assay (ELISA) kits (Axis-Shield, Dundee, Dundee, UK). Serum total Hcy concentrations were measured by sandwich ELISA (µmol L^{-1}).

Acridine orange staining. Acridine orange binding to RNA interferes DNA fragmentation index calculation in sperm chromatin structure assay.[22] The percentage of spermatozoa stained with Acridine Orange were determined by counting 200 spermatozoa per slide under 400× magnification. Red and green sperm could be observed. Green sperms were classified as normal DNA and yellow to red sperms were classified as damaged DNA (D-DNA), (Fig. 1).

Acidic aniline blue staining. As previously described by Hofmann and Hilscher,[23] sperm chromatin quality was studied by aniline blue, as an indicator of significant chromatin/DNA damage. The air-dried fixed smears were stained for 5 min in 5.00% aqueous aniline blue solution (5.00 g aniline blue (Sigma-Aldrich, St. Louis, USA) and 4.00% acetic acid (Sigma-Aldrich, USA) in double distilled water pH = 3.50). The percentage of spermatozoa stained with aniline blue and aniline blue positive (ANBP) were determined by counting 400 spermatozoa per slide under bright field microscope under 400× magnification (Fig. 1).

Statistical analysis. Statistical analyses were performed with SPSS Software (version 24.0; IBM Corp., Armonk, USA). Data were expressed as mean ± standard deviation and the samples were analyzed using repeated-measures analysis of variance. The Bonferroni correction was applied when comparing the percent change in each betaine treatment groups compared to the control. Differences with values of $p < 0.05$ were considered to be significant statistically. Bivariate associations between homocysteine levels and SOD and GPX activities as well as sperm parameters the betaine group during HS were evaluated with Pearson's correlation coefficient.

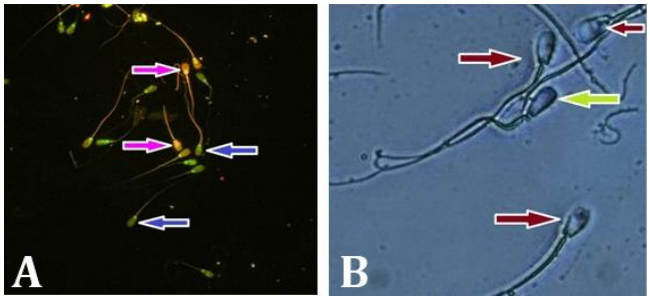

Fig. 1. A) Acridine orange staining for detecting DNA damage of spermatozoa. Indigo arrows show spermatozoa with normal DNA and purple arrows indicate spermatozoa with damaged DNA (100×), **B)** Aniline blue staining to recognize remained histones of sperm chromatin. Dark red arrows indicate normal spermatozoa and the lime one points out the spermatozoid with remained histones (400×).

Results

Mean humidity percentages were 88.00, 92.00, 93.16 and 70.00 from June until September during study period. At that period mean ambient temperatures (bulls keeping hall) were 26.50, 24.02, 22.50, 20.01, respectively. Averages indoors ambient temperature in early summer was significantly higher than in early autumn. The monthly average THI values obtained under THI model in the present study, varied from 66.35: comfort status, in the month of September (T4) to 78.26: alert status, in the month of July (T1). THI values were grouped into four groups: T1: 78.26 (severe HS), T2: 74.44 (moderate HS), T3: 71.94 (moderate to absence of HS), T4: 66.35 (absence of HS), (Fig. 2).

Averages indoors ambient temperature in early summer was significantly higher than in early autumn. Regarding rectal temperature (RT), measurements were ranged from 39.10 to 38.42 ˚C in different classified betaine groups 39.50 to 37.80 ˚C and not different between BET treatment and control groups ($p > 0.05$). The general RT averages during four months of observation were different significantly ($p < 0.05$). Tropical climates with high ambient temperature and humidity might lead to HS (Fig. 3).

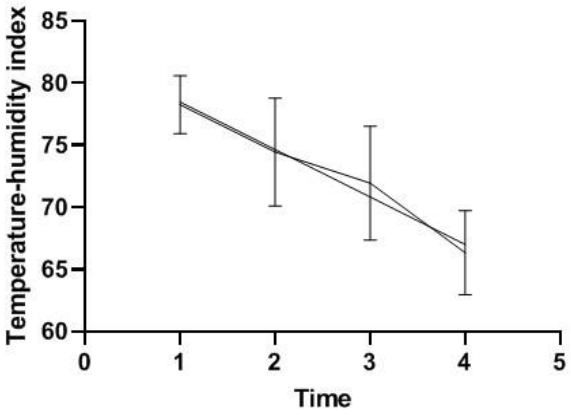

Fig. 2. The monthly average temperature-humidity index values from June to September (T1 to T4).

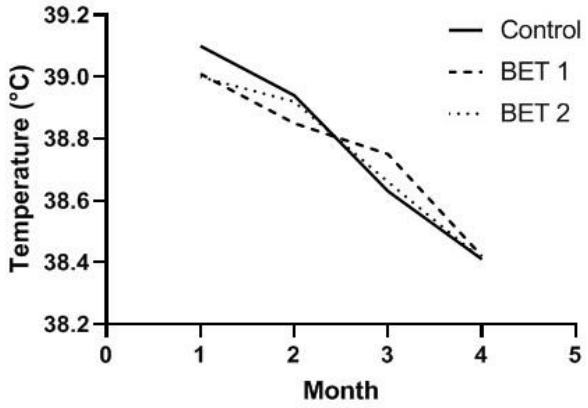

Fig. 3. The rectal temperature from June to September in three groups including control, BET1, and BET2.

Post-thawed semen parameters of fertile bulls were different in the early stage of HS (T1 and T2), late stage of HS (T3) and after periods of HS (T4). The HS had a strong impact on the overall semen parameter that was remarkably improved in time 4. It (T1) significantly reduced sperm viability ($p < 0.05$), sperm progressive motility ($p < 0.05$), CASA parameters of sperm motility including VSL, VCL, LIN, VAP ($p < 0.05$ for all parameters) and significant increase of abnormal sperm morphology ($p < 0.05$) and abnormal sperm head morphology ($p < 0.05$) were demonstrated compared to CP. Despite improvement in many of these parameters, during the late phase of HS (T3), abnormal sperm morphology ($p > 0.05$) and abnormal sperm head morphology ($p < 0.05$) were still significantly different compared to non-heat-stress days. We found no statistically significant difference in the semen volume, sperm concentration, HOST (percentage of swollen spermatozoa, cytoplasmic droplets, mid-piece abnormality, tail defects and some CASA motility parameters (ALH, MAD and BCF between T1 and T2 with CP ($p > 0.05$ for all comparisons), (Table 1).

Serum homocysteine level concentrations, semen antioxidant enzymes (i.e., SOD, GPx), sperm motility, sperm viability, sperm abnormality, sperm membrane integrity (HOS), percentage of normal intact acrosome, DNA fragmentation (using acridine orange staining) and sperm chromatin integrity (using aniline blue staining) when bulls were heat stressed during three months of summer including June (T1), July (T2) and August (T3) in all groups are summarized in Table 2. Under maximal HS (T1), serum homocysteine concentrations reached 16.67 ± 0.09 μmol L^{-1}, however, homocysteine concentrations tended to decrease when body temperature was decreased in T2 and T3 (16.48 ± 0.11 and 15.59 ± 0.14). Hcy concentration was lower in BET2 group ($p < 0.05$) compared to the control group and BET1 group. The supplementation of BET did not provide a significant effect on the level of post-thaw sperm abnormality, sperm membrane integrity, sperm chromatin integrity when bulls were exposed HS.

The percentage of sperm motility was significantly increased in high-dose treated group compared to the control group ($p < 0.05$). Both treated groups also exhibited a significant increase in the percentage of sperm viability during HS period (BET1, $p < 0.05$ and BET2 $p < 0.05$). The HS had also a strong impact on the number of DNA fragmentation of sperm. Dietary betaine supplementation influenced DNA fragmentation of sperm and it was higher in the control group compared to group BET2 (7.80 ± 0.44 *vs* 5.93 ± 0.38; $p < 0.05$). Following the freeze-thawing process, supplementation with BET2 led to lower SOD activity in seminal plasma compared to the control (0.44 ± 0.01 *vs* 0.52 ± 0.01; $p < 0.05$, respectively), but the effect of lower doses of betaine (57.00 mg) did not provide difference on the level of SOD activity when

compared to the controls ($p > 0.05$). There were significant decrease in seminal plasma GPX activity in bulls treated with BET1 and BET2, where the control was 0.08 ± 0.00, BET1 group was 0.07 ± 0.00 and BET2 group was 0.06 ± 0.00 ($p < 0.05$). In the control bulls, HS caused a significant increase in SOD and GPx activities, respectively, from 0.60 to 0.36 and 0.08 to 0.07 U mg^{-1} protein. The SOD activity in T1 (0.60 U mg^{-1} protein; $p < 0.01$) or T2 (0.46 U mg^{-1} protein; $p < 0.05$) was significantly higher than that in T4 (0.36 U mg^{-1} protein). Similarly, there were significant increase in GPx activity in early summer with early autumn: T1 (0.08 U mg^{-1} protein; $p < 0.05$) and T2 (0.07 U mg^{-1} protein; $p < 0.05$) with T4 (0.06 U mg^{-1} protein).

In BET2 groups, high level of plasma homocysteine was correlated with increased DNA fragmentation (DF), abnormal sperm membrane integrity and activities of antioxidant enzymes, GPx and SOD. Also, a significant negative correlation was observed between Hcy and sperm motility and viability (R = 0.35, $p < 0.05$ and R = 0.33, $p < 0.05$). A strong positive correlation was seen between DF rates and the SOD activities (R = 0.51, $p < 0.05$), the correlation between DF rate and GPx was less

Table 1. The impact of hot weather on semen parameters after thawing in heat-stressed and non-heat-stressed condition. Values are expressed as means ± SD.

Parameter	Time			*p*-value	
	Time 1	Time 2	Time 4	$T_1 \times T_4$	$T_2 \times T_4$
Via (%)	46.04 ± 0.81[a]	53.68 ± 0.31	57.13 ± 0.54[a]	0.00	0.53
Pm (%)	38.49 ± 0.53[a]	40.47 ± 0.73	42.37 ± 0.71[a]	0.00	0.64
Morph (%)	18.58 ± 0.34[a]	16.67 ± 1.64[b]	12.74 ± 1.13[ab]	0.00	0.02
HOST (%)	30.27 ± 1.98	31.41 ± 1.44	29.25 ± 1.80	1.00	1.00
Cyt (%)	0.79 ± 0.32	0.88 ± 0.20	0.40 ± 0.11	1.00	0.25
Mid (%)	1.79 ± 0.16	1.68 ± 0.23	1.30 ± 0.18	0.33	1.00
Head (%)	6.57 ± 0.96[a]	6.39 ± 1.21[b]	3.48 ± 0.50[ab]	0.00	0.02
Tail (%)	9.41 ± 0.99	7.70 ± 1.19	7.62 ± 0.91	0.25	1.00
VCL	47.56 ± 1.53[a]	51.05 ± 1.64	55.77 ± 1.90[a]	0.00	0.39
VSL	28.87 ± 0.97[a]	30.98 ± 0.91	34.05 ± 1.15[a]	0.00	0.39
VAP	33.49 ± 0.92[a]	35.47 ± 0.92	38.89 ± 1.19[a]	0.00	0.18
LIN	38.88 ± 0.89[a]	54.74 ± 11.93	44.50 ± 1.15[a]	0.00	1.00
BCF	2.38 ± 1.82	0.76 ± 0.02	0.84 ± 0.02	1.00	0.17
MAD	22.80 ± 0.88	24.18 ± 0.19	24.69 ± 0.82	0.78	1.00
ALH	2.17 ± 0.04	2.27 ± 0.06	2.38 ± 0.06	0.06	1.00

T1: June, severe heat stress (THI values: 78.26). T2: July, moderate heat stress (THI values: 74.44). T4: September, absence of heat stress (THI values: 66.35). Via: Viability. PM: Progressive motility. Morph: Abnormal morphology. HOST: The mean percentage of positive HOST sperm. Cyt: Percentage of cytoplasmic droplets. Mid: Defects of the mid-piece. Head: The mean percentage of spermatozoa with abnormal heads. Tail: Abnormal tail morphology. VCL: Curvilinear velocity. VSL: Straight line velocity. VAP: Average path velocity. LIN: linearity. BCF: Beat cross frequency. MAD: Mean angular displacement. ALH: Amplitude of lateral head.
[ab] Different letters on the same line indicate differences in three time points ($p < 0.05$).

Table 2. Homocysteine concentrations, some other seminal parameters and sperm characteristics measured during summer. Values are expressed as means ± SD.

Parameter	Groups			Time			*p*-value		
	Control	BET1	BET2	T1	T2	T3	Treat	Time	T × G
Hcy (µmol L^{-1})	16.57 ± 0.12[a]	16.16 ± 0.10	16.00 ± 0.10[a]	16.67 ± 0.09[a]	16.48 ± 0.11[b]	15.59 ± 0.14[ab]	0.006*	0.000*	0.020*
D-DNA (%)	7.80 ± 0.44[a]	6.63 ± 0.38	5.93 ± 0.38[a]	7.13 ± 0.25[ab]	6.65 ± 0.22[a]	6.57 ± 0.23[b]	0.012*	0.000*	0.471
ANBP (%)	2.24 ± 0.24	2.11 ± 0.21	2.01 ± 0.21	2.20 ± 0.14[a]	2.15 ± 0.14[b]	2.00 ± 0.11[ab]	0.760	0.003*	0.910
GPX (U mg^{-1} protein)	0.08 ± 0.00[ab]	0.07 ± 0.00[a]	0.06 ± 0.00[b]	0.08 ± 0.00[ab]	0.07 ± 0.00[ac]	0.06 ± 0.00[bc]	0.002*	0.000*	0.501
SOD (U mg^{-1} protein)	0.52 ± 0.01[a]	0.48 ± 0.01	0.44 ± 0.01[a]	0.61 ± 0.01[ab]	0.44 ± 0.01[ac]	0.40 ± 0.01[bc]	0.001*	0.000*	0.743
Via (%)	47.56 ± 1.32[ab]	53.70 ± 1.30[a]	53.43 ± 1.26[b]	46.04 ± 1.17[ab]	53.80 ± 2.03[a]	53.40 ± 2.80[b]	0.005*	0.000*	0.302
Morph (%)	16.51 ± 2.61	13.22 ± 2.26	21.00 ± 2.26	18.58 ± 1.34	16.67 ± 1.64	15.49 ± 1.81	0.066	0.107	0.041*
PM (%)	37.63 ± 0.80[a]	40.02 ± 0.74	41.33 ± 0.77[a]	38.49 ± 0.53	40.47 ± 0.73	40.02 ± 0.79	0.008*	0.091	0.104
HOST (%)	32.86 ± 2.05	30.93 ± 1.77	29.02 ± 1.77	30.27 ± 1.03	31.41 ± 1.41	31.13 ± 2.08	0.377	0.836	0.916
Head (%)	6.72 ± 1.70	3.39 ± 1.47	7.63 ± 1.47	6.57 ± 0.96	6.39 ± 1.21	4.78 ± 1.00	0.123	0.152	0.000

BET1: 57.00 mg of BET kg^{-1} of BW per day, BET2: 114 mg BET kg^{-1} of BW per day. T1: June, T2: July, T3: August, Treat: Treatment, T × G: Treatment × Groups, Hcy: Plasma homocysteine level, D-DNA: Sperm DNA fragmentation, ANBP: Spermatozoa stained with aniline blue, GPX: Activity of seminal plasma glutathione peroxidase, SOD: Activity of seminal plasma superoxide dismutase, Via: viability, Morph: Abnormal morphology, PM: progressive motility, HOST: The mean percentage of positive HOST sperm, and Head: The mean percentage of spermatozoa with abnormal heads.
* indicate significant difference between groups and time on the same row ($p < 0.05$).
[ab] Superscript letters denote significant difference between treatments ($p < 0.05$).

clear (R = 0.32, $p > 0.05$). Moreover, a significant negative correlation was observed between DNA damage and sperm motility as well as morphology (R = –0.037 and R = –0.38). In BET groups, a significant negative correlation was observed between SOD activities and sperm motility ($p < 0.05$, R= –0.43), sperm morphology ($p < 0.05$, R= –0.44) and sperm viability ($p < 0.05$, R= –0.51), while its activity was positively correlated with GPx activity ($p < 0.05$, R= –0.43). GPx activity in testicular fluid concentrations was correlated (R = 0.38) with sperm motility. In contrast, GPx activity was not in correlation with any different sperm parameter. The correlations for each pairing of variables are shown in Table 3.

Discussion

According to the results obtained in this study, the monthly average THI values during summer months indicated that HS contributed significantly to decrease in semen quality in comparison with bulls not exposed to HS. Our findings showed that post-thawed viability, progressive motility, abnormal morphology, abnormal head morphology and CASA parameters (VSL, VCL, LIN, VAP) of cryopreserved bull spermatozoa were affected by HS adversely during the hot summer months, whereas the number of spermatozoa, semen volume, percentage of swollen spermatozoa, membrane integrity, cytoplasmic droplets, Mid-piece abnormality, tail defects, ALH, MAD and BCF were not significantly varied depending on HT.

One of the main factors affected the bull fertility was the DNA fragmentation and chromatin integrity in spermatozoa, which could be associated with HS. The present study indicated that HS increased the activities of SOD and GPx, which were improved with decrease of air temperature. It has been well established that increased scrotal temperatures in the testes lead to an increase of testicular metabolism and oxygen utilization without a corresponding rise in testicular blood flow.[24,25] This has been termed "hypoxia-reperfusion injury". Hypoxic damage generates ROS in tissues after the reestablishment of the normal temperature and tissue reperfusion.[25,26] The HS increases the production of reactive oxygen species and changes in oxidative enzyme activity.[27,28] Moreover, it was reported that antioxidant defense systems in seminal plasma was associated with seasonal changes.[29,30] The generation of ROS during HS in male germ cells may lead to severe DNA oxidative damage and abnormal chromatin condensation.[31] Our data analysis revealed a significantly higher percentage of sperm DNA fragmentation and chromatin damage in warmer months than in cooler months. The results of the present study showed that BET supplements ameliorated oxidative stress status and sperm DNA fragmentation, which increased sperm motility and viability subsequently. Sperm with DNA fragmentation showed significant negative correlations with semen parameters: Viability (R= –0.37, $p < 0.05$) and normal morphology (R = –0.38, $p < 0.05$) were significantly enhanced in the BET2 group. We also found a positive correlation between DF and SOD activities in the BET2 group. The percentage ANBP was calculated and compared among groups, however, the differences were not statistically significant.

Sahin *et al*., exposed Japanese quail to HS and reported greater homocysteine serum concentrations than birds kept at thermoneutral temperature.[32] There are few studies conducted to investigate the Hcy levels in cattle. In a study by Kiliçkap and Kozat, serum Hcy levels in various cow breeds were estimated as follow: 17.44 ± 1.22 µmol L^{-1} in Simmental, 17.04 ± 1.13 µmol L^{-1} in Holstein, and 16.35 ± 1.24 µmol L^{-1} in Montafon.[33] Betaine donates one of its methyl groups to convert homocysteine into L-methionine and increases plasma S-adenosyl-methionine (SAM).[9] Some studies have reported that lower level of seminal SAM was negatively correlated with sperm parameters. Therefore, human sperm motility was evaluated objectively with SAM.[34] Mutation in the methylenetetrahydrofolate reductase (MTHFR) results in hyperhomocysteinemia.

Table 3. Correlation coefficients of sperm quality characteristics in seminal plasma of treated group using Pearson correlation with significant (2-tailed).

Parameter	Hcy	D-DNA	ANBP	GPX	SOD	Via	Morph	PM	HOST	Head
Hcy	1.00	0.35*	0.03	0.51**	0.49**	-0.35*	-0.27	-0.33*	0.12	0.08
D-DNA		1.00	0.05	0.32	0.51**	-0.37*	-0.38*	-0.27	0.16	-0.21
ANBP			1.00	0.07	0.11	-0.27	-0.03	-0.23	0.37*	-0.28
GPX				1.00	0.43**	-0.28	-0.13	-0.38*	0.21	0.20
SOD					1.00	-0.51**	-0.44**	-0.43**	0.11	-0.20
Via						1.00	-0.26	0.62**	-0.28	0.27
Morph							1.00	-0.11	-0.04	0.35*
PM								1.00	-0.10	0.07
HOST									1.00	-0.30
Head										1.00

Hcy: Plasma homocysteine level, D-DNA: Sperm DNA fragmentation, ANBP: Spermatozoa stained with aniline blue, GPX: Activity of seminal plasma glutathione peroxidase, SOD: Activity of seminal plasma superoxide dismutase, Via: viability, Morph: Abnormal morphology, PM: progressive motility, HOST: The mean percentage of positive HOST sperm, and Head: The mean percentage of spermatozoa with abnormal heads. Correlation was significant at the level * $p < 0.05$ and ** $p < 0.01$.

It has been shown that severe MTHFR deficiency in human subsequently leads to production of toxic reactive oxygen metabolites and abnormal spermatogenesis in male that led to homocysteine-mediated DNA damage.[35] It also affected the oxidative status of the reproductive tissue, since homocysteine could mediate DNA damage in spermatozoa.[36]

In the present study, BET supplements improved most important parameters of sperm. However, the differences between all measured parameters were significant only for sperm motility and viability. Consequently, the osmotic properties of betaine had the potential to improve animal performance against HS via preventing increase in vascular permeability and consequent loss of blood plasma water, reduction of epidermal dehydration in animals that sweat under HS affecting and preserving kidney function. Moreover, BET could ameliorate thermal damage to cells by reduction of intracellular energy depletion and protein denaturation.[12] It is possible that the osmolar effects of betaine might be diminished during HS. Further, its effects on the methionine cycle became more critical. The BET acts as enzymatic substrates, co-substrates and co-factors that ultimately lead to improved methyl transfer and reduction in homocysteine levels. Based on previous studies, many of the treatments with improved methionine cycle functionality showed promising effect on improving animal reproductive performance.[16] Dietary supplementation with betaine has been shown to improve reproductive characteristics in mice,[7] bucks,[13] boar,[14] and sows.[16] under HS conditions. It was reported that treatment with betaine significantly improved sperm motility and concentration and decreased lipid per-oxidation products in ethanol-induced oxidative stress of rat testes. Furthermore, the plasma total homocysteine (tHcy) reduction also was shown to affect on betaine as a homocysteine-lowering therapy, antioxidant and methyl donor.[37] In boars, betaine supplementation reduced serum homocysteine concentrations and tended to increase total sperm production in the summer months,[14] which was similar to present study.

Previous findings suggested that increased metabolism after HS might not increase blood flow sufficiently to maintain oxygen consumption (VO_2 or volume of oxygen) facing reduced oxygen, and might become hypoxic. Hypoxia induces cell cycle arrest, oxidative damage to DNA and apoptosis. The increase of ROS following hypoxia has been extensively documented. Moreover, it has been identified as one of the major risk factors which were affected fertilizing potential of spermatozoa. Seminal plasma contains enzymatic ROS scavengers such as superoxide dismutase, glutathione reductase, glutathione peroxidase, glutathione S-transferase and heme oxygenase.[31,37,38] Lipids were largely responsible for membrane structure and fluidity and the and the changes associated with the sperm capacitation process.[39] It has shown that excessive levels of ROS were highly disruptive for sperm function both *in vivo* and in vitro in a dose- and time dependent manner.[31] These effects of BET were suggested to have an impact on the modifications of plasma membrane and intracellular ionic homeostasis.[12] Previous studies found that the antioxidant capacity of betaine enabled it to scavenge free radicals and protect cells from damage in rats.[28] Antioxidant and methyl donor abilities of betaine in oxidative stress in the rat testes showed significantly improved preservation of antioxidant enzymes and its potential to ameliorate the adverse effect of oxidative stress on sperm parameter.[37]

The beneficial effects of betaine on antioxidant enzyme activities were also identified in the present study showing that there were significant decrease in seminal plasma SOD, GPX activity in bulls treated with betaine compared to the controls during HS. It was reported that dietary betaine supplementation was capable of acting as an organic osmolyte or a methyl donor that could improve stress-induced lipid peroxidation.[40] Betaine is reported to contribute to the detrimental effects of structural interactions with membrane lipids.[41] In the present study, GPX activity was decreased in both treated groups, however, the effect of lower doses of BET was not different on the level of SOD activity. The level of activities of antioxidant enzymes (GPx and SOD) were significantly increased in seminal plasma in bull with elevated plasma homocysteine level particularly during hot summer days as well as increased DNA fragmentation rates in the spermatozoa. The activity of GPx and SOD in control group was increased up to 0.08 ± 0.00 mU mL^{-1} seminal plasma and 0.52 ± 0.01 U mg^{-1} protein in seminal plasma. We observed a strong negative correlation with SOD activity and sperm motility, viability and morphology. In the present study, the GPx activity was correlated with decreased sperm motility.

Based on the present findings and compared to findings of similar studies, betaine was shown to bear positive influence of antioxidant and membrane-stabilization on stress- or chemical-induced cellular damage in the liver by restoring both non-enzymatic and enzymatic antioxidants.[42] Significant positive correlations between blood serum SOD and CAT with sperm count and a negative correlation of serum CAT with abnormal morphology and the percentage of dead spermatozoa in both fertile and infertile men were observed.[43]

Contrary to the present study, other study found that no significant differences were observed between immediate and long-term effects of HS considering GPx and SOD activities in epididymal sperm of ram semen.[27] They have hypothesized that GPx was present in the epididymis being oxidized to an inactive state, which became enzymatically active when required and would be faster and more effective than the synthesis of new ones.[44] Recent studies in stallion semen indicated that the specific

activities of CAT, GPX, and GSR were similar between good and poor freezability, and SOD was significantly higher in good freezability ejaculates than in poor.[45] Equal concentrations of SOD, CAT and GPX as simultaneous additives to a stallion semen extender improved preservation of stallion sperm motility and viability during cold storage.[46] Also, positive correlations were found between withstanding oxidative stress during cryopreservation and SOD content in men seminal plasma, which contributes to an increase in the population of viable and rapidly motile spermatozoa after thawing.[47] In Holstein and Jersey bulls, no significant differences were found between GPx and SOD with motility recovery of spermatozoa after thawing.[48] It has also been reported that the seminal activity of SOD was related to total and progressive sperm motilities following cold storage of boar semen. [49]

It could be concluded that daily supplementation of 114 mg BET kg^{-1} of BW betaine during HS decreased plasma homocysteine concentration. Notably, this reduction were associated with lower antioxidant status. Therefore, this finding supported its therapeutic application in the improvement of HS-induced spermatogenesis complications in bulls.

Acknowledgments

We would like to thank the authorities in Iran Simmental Cattle Breeding Center (Amard Dam Tabarestan Company) especially Mr. Heshmat Allah Jamali, CEO of the company. In addition, we sincerely thank the employees of Iran Simmental Cattle Breeding Center, Mr. Masoud Babaei, Mr. Morteza Fani, Mr. Armin Khaki, Mr. Abed Zarghami, and Mr. Adel Alinezhad for their cooperating in semen collection and freezing procedure. The authors would like to thank the Faculty of Veterinary Medicine, Urmia University, Urmia, Iran, for financial support.

Conflicts of interest

None of the authors have any conflicts of interest to declare.

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