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Improvement of Semen Quality in Holstein Bulls during Heat Stress by Supplementing Omega-3 Fatty Acids

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***Abstract*—**The aim of current study was to investigate the changes in the quality parameters of Holstein bull semen during the heat stress and the effect of feeding a source of omega-3 fatty acids in this period. Samples were obtained from 19 Holstein bulls during the expected time of heat stress in Iran (June to September 2009). Control group (n=10) were fed a standard concentrate feed while treatment group (n=9) had this feed top dressed with 100 g of an omega-3 enriched nutriceutical. Semen quality was assessed on ejaculates collected after 1, 5, 9 and 12 weeks of supplementation. Computer-assisted assessment of sperm motility, viability (eosin-nigrosin) and hypo-osmotic swelling test (HOST) were conducted. Heat stress affected sperm quality parameters by week 5 and 9 ($p<0.05$). Supplementation has significantly increased total motility, progressive motility, HOST and average path velocity in the fresh semen of bulls ($P<0.05$).

***Keywords*—**Bull, heat stress, omega-3 fatty acids, spermatozoa.

I. Introduction

BULL fertility is highly important economically in the cattle artificial insemination (AI) industry, since high quality semen is crucial for successful AI. Quality is, however, influenced by many factors such as bull genetic, management, environment and nutrition. Animal environment is affected by climatic factors that include ambient temperature, humidity, radiation, and wind. Extremes in climate alter energy transfer between the animal and its environment and can affect deleteriously reproduction [1]. Heat stress is the state at which mechanisms activate to maintain an animal's body thermal balance, when exposed to intolerable (uncomfortable) elevated temperature [2]. It is likely that heat stress affects reproductive performance both by direct actions on reproduction and by indirect actions mediated through alterations in energy balance [3]. High environmental temperatures tend to have a detrimental effect on semen production and fertility of bulls of the European breeds [4]. Spermatogenesis is impaired, and testosterone is lower during early exposure to hyperthermia [1]. High ambient temperatures (average 25 ◦C), particularly in association with increasing daylight length during the summer months have been demonstrated to result in a reduction of semen quality in goats [5].

Nutrition and feeding practices can have a profound effect on fertility. Lipids are important molecules that serve as a source of energy and are critical components of the physical and functional structure of cells [6]. Omega-3 fatty acids, in particular Docosahexaenoic acid (DHA), are important for sperm membrane integrity, sperm motility and viability, as well as cold sensitivity [7]. There are evidences to suggest that dietary supplementation with omega-3 fatty acids affects reproduction in men and male of different farm animals [8]-[14].

Holstein–Friesian cattle are more affected by heat stress than other European dairy breeds [15]. Since most of the bulls used in AI centers of Iran have been of the Holstein breed developed in temperate areas, there is a need for knowledge of the effects of elevated temperatures on the semen characteristics and fertility of such bulls. Therefore, our goal was to study the changes in the quantity and *in vitro* quality of Holstein bull semen during the period of heat stress and secondly, to investigate the effects of omega-3 fatty acids supplementation in this period.

II. Materials and Methods

A. Bulls and Diets

Experiment was conducted during the expected time of heat stress in Iran (June to September 2009). Twenty Holstein bulls housed in single pens were used for semen collection at Semen Production Center, Karaj, Iran (35°.47' N, 50°.55' E). At the beginning of the experiment the bulls were 2.5-6 years old. They were routinely semen collected three times a week while allowed to false mount several times to provide sexual stimulation prior to each collection. Bulls were randomly assigned to one of two treatment groups (n=10), balanced for bull age; however one of the bulls in the treatment group was removed from the experiment due to poor health condition. Control group were fed a standard concentrate feed with 15%

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of crude protein, and about 3 Mcal/kg metabolizable energy. Treatment group bulls had this standard feed top dressed with 100 g of a commercially available nutriceutical (Optomega 50, Optivite International Limited, Nottinghamshire, UK). The nutriceutical was composed of salmon oil, wheat bran, palm kernel extract, hydrated magnesium aluminosilicate and vitamin E. Nutrient composition of the nutriceutical was as follows: crude protein 4%, crude fat 25% (minimum), crude fiber 3.5%, omega-3 fatty acid 25% (10% DHA, 6% EPA). Two groups of bulls were fed the experimental diets for a total of 12 weeks in order to allow spermatogenesis and maturation of new generation of spermatozoa take place. Water was provided ad libitum for bulls.

B. Semen Collection

Semen was collected using an artificial vagina from the bulls and immediately transferred in to a 37°C water bath. Semen volume was recorded by reading from graduated tubes and sperm concentration was measured using a calibrated photometer (IMV, L'Aigle, France). The total sperm output (volume x concentration) was calculated. Semen was then diluted in a commercial diluent (Bioxcell, IMV, L'Aigle, France), pre-warmed to 37°C, to a final concentration of 40×106 spermatozoa/mL, allowing 5 min for the extender and semen to interact. Afterwards diluted semen was packaged into 0.5 ml straws (Minitube, Germany) and maintained for 6 hours in 4°C before freezing by computer controlled freezing system (Minitube, Germany). After the freezing process, the straws were transferred to a liquid nitrogen tank until subsequent analysis.

Fresh and associated frozen samples were collected after 1, 5, 9 and 12 weeks of feeding omega-3-enriched nutriceutical and sperm quality parameters were analyzed.

C. Calculation of THI

Meteorological data was collected from the weather station in closest proximity to the AI center. Temperature-humidity index (THI) was calculated as follow [16]:

$$THI = (0.8 \times Tdb) + [(RH/100) \times (Tdb - 14.4)] + 46.4$$

Where Tdb is dry bulb temperature in degrees Celsius and RH is relative humidity.

D. Sperm Quality Parameters

An aliquot of semen was placed on a pre-warmed microscope slide and overlaid with a coverslip. The proportion of progressively motile spermatozoa was subjectively evaluated to the nearest 5% under phase-contrast microscope (Olympus, Tokyo, Japan) at X 200 magnification [17]. All the motility tests were performed after viewing several fields by an experienced technician.

In order to computer-assisted assessment of sperm motility, the CASA setup (Animal Version 12.3H-CEROS, Hamilton Thorne Biosciences, Beverly, MA, USA) was pre-adjusted for bovine sperm analysis (Table I). Four microliters of diluted semen were placed in a 20-µm standard count analysis chamber (Leja, Nieuw-Vennep, The Netherlands). The loaded chamber was placed on the thermal plate of the microscope (37.5°C) for 3 min before analysis [18]. Three randomly selected microscopic fields were scanned six times each. The mean of these 18 scans was used for statistical analysis. The following variables were analyzed: total motility (%); progressive motility (%) and average path velocity (VAP, µm/s).

Viability was assessed by the eosin–nigrosin method [19], spermatozoa were considered to be viable when they were not stained. At least 400 spermatozoa were counted on each smear under phase-contrast microscopy at X 1000 magnification and the proportion of viable spermatozoa calculated.

Plasma membrane integrity of fresh and frozen-thawed spermatozoa was assessed using hypo-osmotic swelling test [20]. Hypo-osmotic swelling solution (100 mOsm/kg) was prepared by dissolving 0.49 g of sodium citrate and 0.9 g fructose in 100 ml distilled water. For HOS test, 250 µl of diluted semen was added to 1ml of the pre-warmed HOS solution and incubated at 37°C for 60 min. Following incubation, a 5µl drop from each sample was transferred to a warm, clean microscope slide and covered with a 18 x 18 mm coverslip. This preparation was examined microscopically using a warm stage, X 400 magnification and phase contrast optics. Two hundred spermatozoa were counted per sample and the number of spermatozoa showing characteristic swelling of tail, an indicative of intact plasma membrane, was recorded.

Two straws of the same batch from each treatment were thawed (37°C for 60 s), the semen from each straw pair was transferred in to a microcentrifuge tube, and the subjective sperm motility and hypo-osmotic swelling test were performed with the same procedure described for fresh semen. The post-thaw sperm motility was evaluated using CASA, which involved placing 4µl of semen between the slide and coverslip [21]. The CEROS semen analyzer, with the same setup used for fresh semen, was then employed, and the same variables were analyzed.

TABLE I
SETTINGS FOR THE HAMILTON THORNE CEROS ANIMAL SOFTWARE

Parameter	Setting
Frame rate (Hz)	60
Frames acquired (no.)	30
Minimum contrast	80
Minimum cell size (pixels) 6	6
Medium VAP cut off (µ/s) (MVV)	50
Medium threshold straightness (%)	70
Low VAP cut off (µ/s)	30.0
Low VSL cut off (µ/s)	15.0
Non-motile head size (pixels)	5
Non-motile head intensity	70
Magnification	1.82 x
Video frequency	60
Illumination intensity	2300
Temperature	37°C

E. Statistical Analysis

Statistical Analysis System software (SAS Institute, version 9.1, 2002, Cary, NC, USA) was used for data analysis. Data obtained from the experimental procedure were analyzed using the Shapiro-Wilk (Proc Univariate) test to pre-determine

TABLE II CLIMATOLOGICAL DATA DURING THE EXPERIMENTAL PERIOD

	Ambient temperature (°C)			Relative humidity (%)			THI
	Min	Max	Mean	Min	Max	Mean	
week 1	17.8	33.0	25.4	18	49	31	70.1
week 5	21	38.4	29.7	17	59	32	75.1
week 9	16.0	33.8	24.9	29	81	50	71.6
week 12	15.4	33.0	24.2	25	78	52	70.9

the normality of the residues. Dependent variables that did not meet statistical premises were subjected to angular transformation [Arcsin√X +1] or logarithmic transformation [log (X + 1)]. The original or transformed data were analyzed by ANOVA for repeated measures using the Mixed Procedure of SAS in order to investigate the effects of diets, weeks of the study and the interaction between weeks and diets. In this procedure, bull was assumed as the subject in order to determine the intra animal correlated variance. Baseline values of each trait measured at the first week used as covariate [12]. Results are expressed as least square means (LSM ± S.E.) with a significance level of 5%.

TABLE III CHANGES IN BULL SEMEN PARAMETERS DURING EXPERIMENTAL PERIOD IN CONTROL AND OMEGA-3 SUPPLEMENTED GROUP (LSM±S.E.)

Parameters (unit)	Weeks of collection				Overall
	1	5	9	12	
Semen Volume (ml)					
Control	5.72±0.23	6.28±0.23	5.68±0.24	5.73±0.25	5.85±0.15
Omega-3 supplemented	5.73±0.24	5.76±0.24	5.07±0.24	5.44±0.26	5.50±0.15
Overall	5.73±0.17^{ab}	6.02±0.17^{a}	5.37±0.17^{b}	5.58±0.18^{b}	-
Concentration (10^6 ml^{-1})					
Control	1030.72±34.91	1123.46±34.91	1000.87±36.36	1028.67±37.98	1045.93±26.67
Omega-3 supplemented	1033.50±36.85	1148.25±36.85	1064.67±36.85	1065.63±38.42	1078.01±27.83
Overall	1032.11±25.37^{a}	1135.86±25.37^{b}	1032.77±25.86^{a}	1047.15±26.99^{a}	-
Total sperm output (10^6)					
Control	5695.75±290.80	6879.50±290.80	5480.68±305.64	5716.12±321.87	5943.01±197.54
Omega-3 supplemented	5726.30±307.01	6495.82±307.01	5221.85±307.01	5663.45±323.29	5776.85±205.01
Overall	5711.02±210.94^{a}	6687.66±210.94^{b}	5351.26±215.85^{a}	5689.79±227.43^{a}	-
Viability (%)					
Control	64.67±4.76	50.35±5.71	54.26±5.22	59.25±4.55^{A}	57.13±3.04
Omega-3 supplemented	57.14±4.89	53.15±5.51	58.23±5.67	73.64±4.89^{B}	60.54±3.19
Overall	60.91±3.35^{ab}	51.75±3.86^{a}	56.24±3.70^{a}	66.44±3.25^{b}	-
Subjective Motility (%)					
Control	61.73±1.52	62.88±1.52	60.58±1.52	57.13±1.57	60.58±1.21
Omega-3 supplemented	61.58±1.60	61.75±1.60	60.72±1.60	59.90±1.66	60.99±1.27
Overall	61.66±1.10^{a}	62.31±1.10^{a}	60.65±1.10^{ab}	58.52±1.14^{b}	-
Subjective post-thaw Motility (%)					
Control	37.84±1.96	36.59±1.96	39.76±2.09	39.04±2.09	38.31±1.24
Omega-3 supplemented	37.96±1.95	38.20±1.85	38.76±1.85	30.03±1.95	38.49±1.16
Overall	37.90±1.38	37.40±1.34	39.26±1.39	39.04±1.43	-

^{a,b,c}Denote differences ($p < 0.05$) in a same row.
^{A,B,C}Denote differences ($p < 0.05$) in a same column.

III. RESULTS

The Climatological data during the experimental period and the regarding calculated THI are shown in Table II. Calculated THI were 70.1, 75.1, 71.6 and 70.9 on week 1, 5, 9 and 12 of experiment, respectively. It can be inferred that the bull had

experienced a mild to moderate heat stress by week 5 to 9 of the study. Changes in bull semen parameters during experimental period in control and omega-3 supplemented group are shown in Table III. Heat stress indeed affected the sperm quality parameters. By week 5 of collection semen volume, sperm concentration and as a consequence total sperm production were higher compared to the other weeks (P>0.05). However, viability and subjective motility of fresh semen was higher on week 12 (p<0.05). The proportion of viable spermatozoa increased (P<0.05) in the ejaculates collected from omega-3-fed bulls compared to the control after 12 weeks of feeding trial.

TABLE IV CHANGES IN CASA PARAMETERS OF FRESH BULL SEMEN IN CONTROL AND OMEGA-3 SUPPLEMENTED GROUP (LSM±S.E.)

Parameters (unit)	Weeks of collection				Overall
	1	5	9	12	
Total Motility (%)					
Control	81.09±1.93	84.99±1.93	75.95±2.04^{A}	83.95±2.04	81.49±1.16
Omega-3 supplemented	78.01±2.04	86.45±2.04	84.90±2.04^{B}	88.05±2.16	84.34±1.21
Overall	79.55±1.40^{a}	85.72±1.40^{b}	80.42±1.43^{a}	85.98±1.47^{b}	-
Progressive Motility (%)					
Control	58.50±1.54	59.50±1.54	58.15±1.61^{A}	62.40±1.54^{A}	59.64±1.14
Omega-3 supplemented	57.91±1.73	61.04±1.73	64.04±1.73^{B}	67.14±1.82^{B}	62.53±1.28
Overall	58.21±1.15^{a}	60.27±1.15^{ab}	61.09±1.17^{b}	64.77±1.19^{c}	-
Average path velocity (μm/s)					
Control	128.99±2.49	128.99±2.49	118.06±2.63^{A}	124.38±2.63	125.11±1.31
Omega-3 supplemented	128.21±2.63	129.50±2.63	126.28±2.78^{B}	128.61±2.78	128.15±1.39
Overall	128.60±1.81^{a}	129.24±1.81^{a}	122.17±1.91^{b}	126.50±1.91^{ab}	-

^{a,b,c}Denote differences (p < 0.05) in a same row.
^{A,B,C}Denote differences (p < 0.05) in a same column.

TABLE V CHANGES IN CASA PARAMETERS OF POST-THAW BULL SEMEN IN CONTROL AND OMEGA-3 SUPPLEMENTED GROUP (LSM±S.E.)

Variables (unit)	Weeks of collection				Overall
	1	5	9	12	
Total Motility (%)					
Control	45.30±4.48	31.56±4.98	44.68±4.70	40.09±4.48	40.41±1.99
Omega-3 supplemented	40.33±4.72	29.11±4.72	41.89±4.72	45.41±4.99	39.18±2.02
Overall	42.81±3.25^{a}	30.34±3.43^{b}	43.28±3.33^{ac}	42.75±3.35^{ac}	-
Progressive Motility (%)					
Control	31.82±3.07	27.63±3.07	33.44±3.23	31.39±3.43	31.06±1.51
Omega-3 supplemented	29.57±3.23	28.19±3.24	35.27±3.67	34.25±3.43	31.81±1.60
Overall	30.69±2.23	27.91±2.23	34.36±2.45	32.82±2.43	-
Average path velocity (μm/s)					
Control	111.74±4.57	118.93±4.57	123.56±4.82	114.82±4.57	117.27±2.17
Omega-3 supplemented	111.83±4.82	113.35±4.82	127.55±4.82	122.77±4.82	118.87±2.25
Overall	111.79±3.32^{a}	116.14±3.32^{ab}	125.56±3.40^{b}	118.80±3.32^{ab}	-

^{a,b,c}Denote differences (p < 0.05) in a same row.

Changes in CASA parameters of fresh and post-thaw bull semen in control and omega-3 supplemented group are presented in Table IV and Table V. Dietary supplementation did not affect the subjectively assessed motility in both fresh and post-thawed semen (P>0.05). However, when motility was assessed by CASA, both total and progressive motility of fresh semen were higher after 9 weeks of supplementation (P<0.01 and P<0.05 respectively) between two groups (Table 3). Similarly, average path velocity of fresh semen were significantly higher in omega-3-fed group in comparison with control (P<0.05).

Semen cryopreservation was indeed found to affect sperm kinematics. The estimated losses related to the cryopreservation process were 50.41% and 53.55% for total motility and 47.93% and 49.13% for progressive motility in control and omega-3-fed groups, respectively. The post-thawed sperm motility parameters assessed by CASA did not significantly differ between two groups. However, the post-thawed VAP were higher in omega-3-fed bulls after 9 weeks

of supplementation (P>0.05).

Changes in the proportion of HOST-positive spermatozoa in control and omega-3 supplemented bulls are shown in Table VI. Diet enriched with Omega-3 significantly increased the percentage of HOST-positive spermatozoa in fresh semen (P<0.03), wherein two groups demonstrated the largest difference after 9 weeks of nutriceutical supplementation (P<0.01). On the other hand, the effect of diet on post-thawed HOST was not significant (P=0.10), however there was an increasing tendency in the proportion of HOST-positive spermatozoa.

IV. Discussion

The aim of our work was to study the changes in the quantity and *in vitro* quality of Holstein bull semen during the period of heat stress and secondly, to investigate the effects of omega-3 fatty acids supplementation in this period. Therefore, this experiment was conducted during the probable time of heat stress (June to September) in Iran. It can be inferred from the meteorological data and calculated THIs that by week 5 of the experiment, the highest heat stress has occurred. When the THI exceeds 72, cattle are affected adversely [22]. Subsequently, there was a significant decreasing tendency in the semen volume, sperm concentration and total sperm production until week 9 of study. A testicular temperature that is below body temperature is known to be essential for the production of fertile spermatozoa [23]. Higher ambient temperature may result in increased testicular temperatures and thus decrease semen quality. Spermatogenesis and epididymal maturation in bull take about 65 days [24]. The results for ejaculate volume are in accordance with Meyerhoeffer et al. [25]. In their experiment in which AI bulls were exposed to heat, the authors observed a decrease in ejaculate volume during the first 6 weeks of heat stress. Similar to the present results, in a previously report [25] a decrease of the percentage of motile sperm 2 weeks after exposure to heat was found. They assumed that heat stress might not affect epididymal sperm while an impact during spermatogenesis exists.

Table VI Changes in proportion of HOST-positive spermatozoa in control and omega-3 supplemented bulls (LSM±S.E.)

Parameters (unit)	Weeks of collection				Overall
	1	5	9	12	
Fresh HOST (%)					
Control	55.55±1.92	57.24±1.92	51.80±2.02^{A}	64.58±2.03	57.29±1.08^{A}
Omega-3 supplemented	55.34±2.03	60.34±2.03	59.73±2.03^{B}	69.46±2.15	61.22±1.12^{B}
Overall	55.44±1.40^{a}	58.79±1.40^{a}	55.76±1.43^{a}	67.02±1.48^{b}	-
Post-thaw HOST (%)					
Control	22.57±2.18	23.79±2.18	31.85±2.18	33.96±2.18	28.04±1.24
Omega-3 supplemented	23.62±2.18	24.93±2.18	31.81±2.18	37.52±2.31	29.47±1.25
Overall	23.09±1.54^{a}	24.36±1.54^{a}	31.83±1.54^{b}	35.74±1.58^{b}	-

^{a,b,c}Denote differences (p < 0.05) in a same row.
^{A,B,C}Denote differences (p < 0.05) in a same column.

In this study feeding an omega-3 enriched nutriceutical improved the motion characteristics of fresh sperm assessed by CASA. Total motility, progressive motility and average path velocity of nutriceutical-fed bulls were significantly higher than the control group after 9 weeks of feeding trial. Our results are consistent with the results of Conquer [8] in human, Rooke et al. [12] in boar, Dolatpanah et al. [26] in goat and Towhidi et al. [27] in sheep who reported a significant correlation between dietary omega-3 supplementation and the number of motile spermatozoa. However, in contrast, studies in boar [11], stallion [13],[14] and turkey [10] found no evidence of a positive effect of dietary omega-3 fatty acids supplementation on fresh semen motility. DHA is the major long chain PUFAs found in the phospholipids of the spermatozoa in the mammals [28]. Conner et al. [29] observed that almost all of the DHA in monkey sperm is located in the tail, while the head region phospholipids contain very low amounts of DHA. In their study, it was estimated that surprisingly the tail contained 99% of the total DHA of spermatozoa. To a certain extent, it can be inferred that high content of this long chain polyunsaturate in sperm tail may contribute to the flagellar action and bending movement required for motility. DHA acyl-chain in phospholipids bilayers is highly flexible and can rapidly convert between an extended and a looped conformation. This confers springy quality to the membrane, allowing it to accommodate and recover from compressive forces in the lateral plane of the bilayer. Membranes with a high content of DHA in their phospholipids are, therefore, distinguished by high levels of flexibility, compressibility, deformability, and elasticity [30]. Hence, it can be suggested that DHA may have an important role in the physiological and molecular mechanisms controlling the spermatozoa membrane fluidity.

Dietary supplementation did not enhance semen volume, sperm concentration and total sperm production which are in agreement with the results of Adeel et al. [31] in buffalo,

Cerolini et al. [32] in chicken and Zaniboni et al. [10] in turkey. However, feeding a source of omega-3 fatty acids leads to a higher sperm concentration and total production in boar [12] and stallion [13,14]. Different results reported on the effect of DHA on sperm concentration and total production might be related to the proportion of DHA and DPA (Docosapantaenoic acid C22:5n-6) in semen of boar and stallion. The semen of boars has very high levels of DPA and the semen lipid profile of stallions is similar to that of the boar [12,13]. Quite the opposite, DHA is the predominant PUFA in bull spermatozoa whereas DPA is extremely lower [33].

We observed an increasing tendency in the percentage of swelling with the hypo-osmotic test throughout the experiment. It can be suggested that feeding omega-3 enriched nutriceutical may increase the incorporation of DHA in to the principle piece, facilitating sperm membrane stability against hypo-osmotic solution. On the other hand, increases in the number of viable cells is again similar to the results obtained in boar [12] and Turkey [10], but in contrast with results reported in stallion [13]. Results may vary according to the method applied to assess sperm plasmalemma. Brinsko et al. [13] used flow cytometry for evaluating viable cells but the others assessed viability by means of eosin-nigrosin. Despite the suggested structural role of PUFA in plasmalemma of sperm, DHA incorporation in to spermatozoa might enhance a series of action lead to prevention of early apoptosis as it has been seen in cultured neurons and retinal photoreceptors [34]. This may promote higher number of viable spermatozoa in the ejaculate.

It can be concluded that heat stress has detrimentally decreased sperm quality parameters in Holstein bulls. Moreover, dietary omega-3 supplementation or its precursors, improve in vitro quality and motility parameters of fresh semen assessed by CASA in Holstein bulls. However, this effect was not obvious in frozen-thawed semen.

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