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HBP Concentration Affects *in vitro* Acrosome Reaction and Conception Rate in Murrah Buffalo Bulls: Interrelationship among Sperm function tests

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ABSTRACT

The aim of this study was to identify relationship between heparin binding proteins (HBP) concentration and *in vitro* acrosome reaction / conception rate / sperm function tests in murrah buffalo bulls. Frozen-thawed semen of 31 bulls was analyzed for HBP concentration, in vitro acrosome reaction, acrosome integrity, DNA integrity and lipid peroxidation. Conception rate of 10 bulls (21-30) was also obtained. Relationship between HBP and *in vitro* acrosome reaction / conception rate was evaluated. Inter-relationship among sperm function tests was also observed. A considerable variation in motility (PTM), DNA integrity, acrosome damage (fully damaged: FDA, partially damaged: PDA), *in vitro* capacitation / acrosome reaction and LPO was observed among 31 tested buffalo bulls after thawing during the present study. A significant positive correlation was observed among various sperm parameters. Thirty one bulls were divided into G-1 (> 40 % AR) and G-II (≤ 40 % AR). There was no difference in HBP concentration, PTM and DNA damaged sperms among the two groups. Percentage of spermatozoa with partially, fully damaged and acrosome reacted spermatozoa was significantly ($p \leq 0.05$) higher in G- I as compared to G-II. MDA level (μmoles / 10^9 spermatozoa) was non-significantly ($p \geq 0.05$) higher in G-II as compared to G-I. Ten bulls (22-31) were divided into two groups; G-I (> 35 % conception rate) and G-II (< 35 % conception rate). Concentration of HBPs was significantly ($p \leq 0.05$) higher in G-I as compared to G-II. Percentage of acrosome reacted spermatozoa was also significantly ($P \leq 0.05$) higher in G-I than G-II. Therefore, HBP concentration in spermatozoa may be useful in predicting the fertility of buffalo bull semen.

Keywords: Conception rate, HBP, In vitro acrosome reaction, Sperm function tests

Efficiency of reproduction is the most important factor that affects overall efficiency of production in most of the domestic animals. Reduction in reproductive efficiency results in great economic loss that indicates the importance of evaluation of fertility while selecting males and females for live stock breeding operations. In bulls, breeding sound evaluation (BSE) is done to select bulls as potential satisfactory breeders. These tests consist of assessing scrotal size and sperm parameters along with general health of the animal. However, bulls that pass BSE, still vary in actual fertility potential (Bellin *et al.,* 1998). Basic laboratory tests such as sperm concentration, motility, morphology and freezability are important but of limited value in predicting fertility owing to subjective errors (Salisbury *et al.,* 1985). Computer-assisted semen analysis (CASA) provides an objective assessment of various motion parameters (Arman *et al.,* 2006), however, differences in CASA based sperm motility assessment due to wide variability in machine parameterization renders it unreliable for fertility prediction

(Rodriguez-Martinez, 2007). Other functional tests, viz. viability, hypo-osmotic swelling test (HOST), chromatin structure, induction of capacitation / acrosome reaction, zona free hamster egg penetration assay and binding of spermatozoa to oocyte have been conducted (Clay and McDaniel, 2001; Petrukina *et al.,* 2007), but inconsistent results makes these tests unreliable for fertility prediction (England and Plummer, 1993).

The HBP are produced by male accessory sex glands, secreted into seminal fluid and upon ejaculation bind to the sperm. The HBP were found to improve sperm motility, cervical mucus penetration (CMPT) and HOST at pre-freeze stage but after freezing drastically reduced these characteristics of buffalo cauda spermatozoa (Harshan *et al.,* 2006). HBP protect sperm from stress of freezing and thawing and maintained intracellular protein homeostasis (Shi *et al.,* 1998). Being a highly differentiated cell, sperm has minimal transcriptional and translational activity and cannot synthesize new heparin (Medeiros *et al.,* 2002). Heparin binding proteins are hypothesized to mediate capacitation by binding to sperm at the time of ejaculation and potentiate the effect of heparin. In bulls, a series of HBPs ranging from 15-40 kDa possess a high affinity to bind heparin. The presence of specific HBP on sperm indicated affinity of sperm to heparin, subsequent ability of sperm to undergo acrosome reaction and thus the fertility potential of a bull (Singh *et al.,* 2013). HBP also protected sperm from lipid peroxidation during cryopreservation (Kumar *et al.,* 2008; Patel *et al.,* 2016). This indicated that HBP play an important role in the survival of sperm during freezing and thawing. Therefore, study was aimed to relate HBP concentration to in vitro acrosome reaction, conception rate and sperm function tests in murrah buffalo bulls.

MATERIALS AND METHODS

Procurement of semen samples

Study was carried out on 31 buffalo bulls. Forty mini straws (0.25 ml) per bull were procured from Animal husbandry department, Rauni, Patiala (21 bulls) and semen freezing lab, Directorate of livestock farms, Guru Angad Dev veterinary and Animal Science University, Ludhiana (10 bulls). Data for first service conception rate for 10 bulls was also obtained from Dairy farm, Directorate of livestock farms, Guru Angad Dev veterinary and Animal Science University, Ludhiana, Punjab, India.

Chemicals and Reagents

All AR grade chemicals of Sisco Research Laboratories, Sigma and BR Biochem were used for this study. Distilled water (DW) from Milliopre purification system (RO/Synergy) was used for the preparation of reagents.

Quantitative ELISA for HBP concentration in spermatozoa

ELISA for HBP concentration was done in duplicate. High binding 96 U bottom wells ELISA plate (BR BIOCHEM, Life Sciences) with 100 µl of poly-L-Lysine (MP biomedicals) was incubated at 37°C for 1 hr. Washed the wells twice with 200 µl of PBS. Then coated each well with 100 µl (5µg / ml) of capture antibody and incubated at 4°C for overnight. Washed the plate thrice with wash buffer and blocked the plate with 200 µl of 0.1% BSA for 1 hrs at room temperature. Repeated the washing step and incubated the plate with 100 µl of 1:1 sperm extracts, 25-200 µg of HBP standard (purified from buffalo bull seminal plasma by affinity chromatography, SP-HBP) and PBS (negative control) for 2 hrs at room temperature. Again washed the plate thrice with wash buffer and added 100 µl of 1:500 diluted anti – HBP (raised in rabbit against purified SP-HBP). Incubated the plate for one hr at room temperature, washed thrice with wash buffer and added 100 µl of 1:10000 diluted HRP conjugated goat anti rabbit IgG. Plate was again incubated for 30 min at room temperature. Washed the plate with PBST thrice and poured 100 µl of OPD-H_2O_2

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and incubated for 20 minutes in dark. After 20 minutes, the reaction was stopped using H_2SO_4 (5 N). The absorbance was measured using an ELISA reader (Tecan) at 492 nm. Concentration of HBP in sperm extracts was calculated from the standard curve.

Sperm function and fertility tests: Al tests were carried out in triplicate.

Acrosome integrity (Ward and Storey, 1984): Acrosome damage was assessed by Chlortetracycline Cysteine Stain (CTC). Twenty five µl of sperm suspension was mixed with 25 µl of 750 µM CTC solution in 20 mM Tris HCl, 130 mM NaCl and 5 mM Cysteine, incubated at 37ºC for 30 secs. Added 100 µl of 12.5% paraformaldehyde and one drop of 0.22 M DABCO. A drop of sperm suspension was placed on a glass slide, covered with cover slip and observed under fluorescence microscope (Olympus) at 400 X using blue filter at 480 nm. Green fluorescence on acrosome, a band between acrosome and equatorial segment and on post-acrosomal cap was observed in spermatozoa with normal, partially damaged and completely damaged acrosome, respectively.

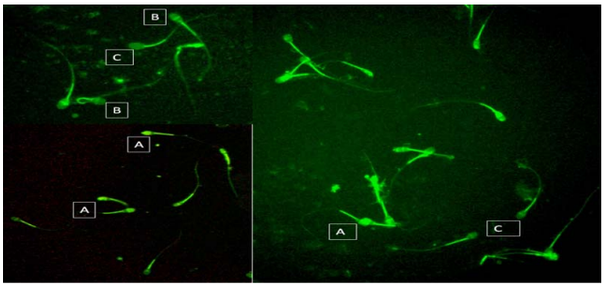

Fig. 1: Various stages of acrosome damage in frozen-thawed semen of buffalo bulls. A: Normal sperm (fluorescence on acrosome), B: Partially damaged (ring of fluorescence at the base of acrosome, C: Fully damaged (No fluorescence on acrosome).

DNA integrity (Lui and Baker, 1992): The acridine orange staining stock solution was prepared by adding 6 mg of AO in 1 ml of DW and stored in the dark at 4°C. Solution A was prepared by mixing 1 mM sodium EDTA, 0.15 M NaCl, 0.3 M $Na_2HPO_4.7H_2O$ and 0.1 M citric acid in 50 ml of DW and pH was adjusted to 6.0. Solution B was prepared by adding 0.1% Triton-X-100, 0.08 N HCl and 0.15 M NaCl in 25 ml of DW. The working AO solution was prepared fresh by adding 3 µl of AO stock solution to 5 ml of solution B and stored in the dark at room temperature until used. Washed frozen-thawed semen twice with phosphate buffer solution (PBS) and pellet was suspended in 0.5 ml of PBS. Added 200 µl of sperm suspension to 400 µl of solution B and mixed gently for 30 seconds. Then added 1.2 ml of ice cold solution A, mixed gently and equilibrated for 15 minutes. Finally, 10 µl of AO was gently mixed with semen placed on a glass slide and covered with coverslip. About 200 spermatozoa were evaluated under an epifluorescent microscope (400 X). The heads of the sperm cells with normal DNA integrity (double stranded) emitted green fluorescence, whereas those with denatured or single stranded DNA had orange / yellow fluorescence (Fig. 1). The slides were evaluated within one hour after staining.

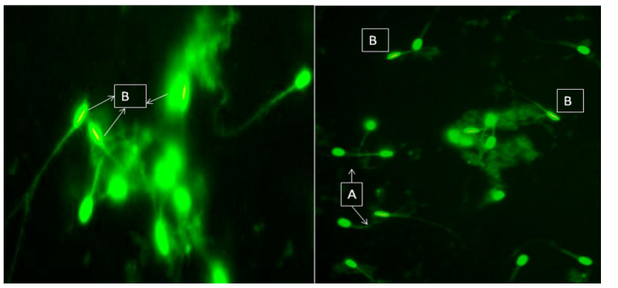

Fig. 2: DNA integrity of spermatozoa in frozen-thawed semen. A: Spermatozoa with double stranded DNA, B: Spermatozoa with denatured DNA.

***In vitro* Capacitation / Acrosome reaction (Yanagimachi 1994):** (i) *Basic TALP medium* (92.9 mM NaCl, 4 mM KCl, 25.9 mM $NaHCO_3$, Na_2HPO_4, 10 mM $CaCl_2.2H_2O$, 0.5 mM $MgCl_2.6H_2O$, 1.3 mM sodium pyruvate, 7.6 mM sodium lactate and 20 mM HEPES. All ingredients were dissolved in DW, final volume was made to 100 ml and pH was set at 7.4. (ii) *Energy medium:* (Basic TALP medium

supplemented with 0.09% glucose, 0.6% bovine serum albumin (BSA), 50 µg / ml gentamycin and 10 µg / ml heparin). Three straws per bull were thawed at 37°C in 5 ml microfuge tube and washed twice with the basic TALP medium (2 ml) by centrifuging at 1000 rpm for 5 minutes. The sperm suspension was re-suspended in the energy medium (0.5 ml) to a final concentration of 200 × 10^6/ml spermatozoa and incubated at 37°C for 3-4 h. Semen was checked for motility and capacitation status after every one hour. A smear was also prepared after every one hour, stained with giemsa and assessed for acrosome reaction. At least 200 spermatozoa were counted from each slide and percentage of acrosome reacted spermatozoa was calculated (Fig. 2).

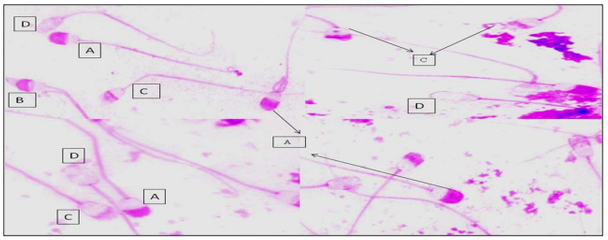

Fig. 3: Various stages of capacitation and acroosme reaction during in vitro capacitation of frozen-thawed semen. A: Un-capacitated, B: Capacitated with condensed acrosome, C: Partially shedded acrosome, D: Completely shedded acrosome.

Lipid Peroxidation (LPO, Buege and Steven, 1978): An aliquot of 0.2 ml of frozen-thawed semen was incubated with 0.2 ml of 150 mM Tris HCL (pH 7.1) at 37°C for 20 minutes. Then 1.0 ml of 10 % TCA and 2.0 ml of 0.375% TBA were added and kept for 20 minutes in the boiling water bath. Mixture was centrifuged at 5000 rpm for 15 min, supernatant was taken out and absorbance was taken at 532 nm. The molar extinction coefficient for MDA was calculated as below:

$$\text{MDA, µmole}/10^9\text{spermatozoa} = \frac{\text{O.D} \times \text{vol of assay mixture}}{\text{Extinction coefficient} \times \text{vol of sample}}$$

First service conception rate: First service conception rate for 10 bulls (22-31) was taken from the dairy farm, directorate of live stock farms, GADVASU, Ludhiana. It was calculated on the basis of 20 inseminations per bull.

Analysis of data: Thirty one bulls were grouped on the basis of capacitaion / acrosome reaction status and compared for HBP concentration, acrosome integrity, DNA integrity, LPO and *in vitro* acrosome reaction. Correlation and regression equation was calculated among various sperm function tests and rate of *in vitro* acrosome reaction and fertility. Bull numbers 22-31 were grouped on the basis of conception rate and compared for HBP concentration, acrosome integrity, DNA integrity, LPO, capacitation / acrosome reaction and conception rate.

Statistical analysis: Significant differences among the two groups were tested by paired 't' sample test using SPSS 16 for windows software. A linear regression model was applied to the relationship among semen traits. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Quantitative analysis of HBP in buffalo bull spermatozoa by ELISA: Quantitative ELISA indicated an average HBP content of 2.04 ± 0.098 mg /10^9 spermatozoa in 31 tested buffalo bulls (Table 1). Overall mean values of HBP were recorded as 2.61 mg / ml in Murrah buffaloes by Arangasamy *et al.* (2005). HBPs were in the range from 0.82 ± 0.04 – 3.22 ± 0.03 mg / 10^9 spermatozoa among 31 bulls tested during the present study. It revealed a high variation in sperm HBP content from bull to bull. Nauc and Manjunath (2000) also were of the opinion that the variation in measured BSP concentrations was high from bull to bull and also from ejaculate to ejaculate within bull. HBP concentration range from 1.47 to 2.61 mg/ml was also earlier reported in buffalo (Singh *et al.,* 2012).

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Table 1: Mean ± SE and range of sperm parameters in frozen-thawed buffalo bull semen

Sperm parameter	Mean ± SE	Range
HBP (mg / 10^9) spermatozoa	2.14 ± 0.098	0.82– 3.22
Motility (%)	59.0 ± 0.69	50.0 ± 0.0 – 62.5 ± 2.5
Spermatozoa with fully damaged acrosomes (%)	12.04 ± 0.72	4.33 ± 0.88 – 18.5 ± 0.87
Spermatozoa with partially damaged acrosomes (%)	26.72 ± 1.36	16.0 ± 0.0 – 53.66 ± 2.08
Spermatozoa with denatured DNA (%)	25.62 ± 1.61	11.5 ± 1.32 – 39.0 ± 2.08
In vitro acrosome reaction (%)	42.90 ± 1.33	32 ± 1.1 – 60.5 ± 1.44
MDA (μmoles MDA / 10^9 spermatozoa)	141.48 ± 9.96	57.46 ± 8.24 – 216.67 ± 18.78

Sperm Function Tests: A considerable variation in motility (PTM), DNA integrity, acrosome damage (fully damaged: FDA, partially damaged: PDA), in vitro capacitation / acrosome reaction and LPO was observed among 31 tested buffalo bulls after thawing during the present study (Table 2). The variation in sperm parameters after thawing was in accordance with the studies reported by Scholamy *et al.* (2009) and Mahmoud *et al.* (2015) on buffaloes. An average post thaw sperm motility of 59.0 ± 0.69% was observed, which ranged from 50 ± 0.0 to 62.5 ± 2.5%. Mahmoud *et al.* (2015) observed post thaw motility in the range of 39.33 – 45.87% in buffalo bull semen.

The presence of intact acrosome is pre-requisite for fertilization and is highly correlated with fertility of frozen semen (Medeiros *et al.,* 2002). An average percentage of spermatozoa with FDA and PDA was 12.04 ± 0.72, 26.72 ± 1.36, respectively. But it ranged from 16.0 ± 0.0 – 53.66 ± 2.0 and 4.33 ± 0.88 – 18.5 ± 0.87 % among the bulls, respectively. Kumar *et al.* (2016) reported significant ($p<0.05$) acrosome damage during freezing-thawing process in buffalo bulls. The overall < 20% acrosome damage was reported during cryopreservation in buffalo (Rasul *et al.,* 2001 and Kumar *et al.*, 2016). But, acrosome damage observed in ram (45-65%, Soylu *et al.,* 2007), goat (38-43%, Chauhan *et al.,* 1994), cattle (20.1%, 26%, Azam *et al.,* 1998, Zodinsanga *et al.,* 2015) and buffalo (16.6 ± 8.3 - 55.8 ± 2.0, Bansal and Cheema, 2014) was close to that observed in buffalo during the present study. Bansal *et al.* (2014) also revealed non-significant ($p\geq0.05$) differences in acrosome damage among the bulls.

Spermatozoa with single stranded DNA varied from 11.5 ± 1.32 to 39 ± 2.08% after freezing-thawing of buffalo bull semen. But, overall average spermatozoa with denatured DNA were 25.6 ± 1.61%. It has been reported that chromatin integrity was affected by freezing (Mahmoud *et al.,* 2015, Mukhopadhyay *et al.* 2011). But Martin *et al.* (2004) were of the opinion that cryopreservation of bull semen had no effect on sperm chromatin stability or DNA integrity. The presence of sperm nuclear chromatin damage cannot be accepted at levels greater than 15-20% of spermatozoa (Barth and Oko, 1989) in fresh semen. Therefore, an acrosome damage of 25.6 ± 1.61% in frozen thawed buffalo semen seems to be within permissible limits during the present study.

Incubation of washed frozen-thawed spermatozoa in TALP supplemented with 0.6% BSA, 0.25% glucose and 10 μM resulted in 14.69 ± 0.58% acrosome reacted (AR) spermatozoa after one hour. A gradual increase in percentage of AR spermatozoa from 14.69 ± 0.58% to 42.79 ± 1.30%) was observed till 3 hrs of incubation (Table 3). Mahmood *et al.* (2007) also incubated frozen-thawed buffalo bull spermatozoa for 30 min in TALP supplemented with 50, 100 and 200 μg/ ml heparin and achieved 37 – 48% AR. Frozen-thawed bovine and buffalo spermatozoa

incubated for 30 min in TALP supplemented with 5 IU and 10 µg / ml heparin resulted in 10% and 20% AR, respectively (Pereira *et al.,* 2000 and Kitiyanant., 2002).

MDA production / 10^9 spermatozoa ranged from 57.46 ± 8.24 to 216.67 ± 18.78 µ moles among 31 tested bulls. But average MDA production (µmoles / 10^9 spermatozoa) was 141.48 ± 9.96 in frozen-thawed buffalo bull semen. Bansal *et al.* (2014) reported 874.13 µmoles / mg protein / ml of MDA production after freezing-thawing of buffalo bull spermatozoa. They also observed non-significant ($p \geq 0.05$) differences in LPO among the bulls. Chatterjee and Gagnon (2001) and Park *et al.* (2003) observed an increased production of ROS during the process of freezing-thawing of semen. Cryopreservation and thawing alone or in combination are likely to induce membrane damage, which is measured by degree of lipid peroxidation of polyunsaturated fatty acids (PUFAs) in cell membrane by free radicals (Bell *et al.,* 1993). Neild *et al.* (2003) also stated that damage to the spermatozoa during freezing-thawing process is caused by lipid per-oxidation, which reduces membrane integrity. It was also observed by Fraczek *et al.* (2001) that polyunsaturated fatty acids (PUFAs) present in the sperm membranes are susceptible to ROS attack, which causes lipid peroxidation during cryopreservation of semen.

Correlation among various sperm parameters of frozen-thawed buffalo bull semen: The correlations among various sperm parameters and linear regressions are shown in Table 2. A significant positive correlation was observed among various sperm parameters. However, correlation between in vitro AR X LPO was nearly zero. Correlation between HBP X LPO; AR X PDA; PDA X PTM and LPO X DNA damaged sperms was higher than that between HBP X AR / PDA / FDA / DNA damaged sperms / PTM; PDA X FDA, FDA X DNA damaged sperms; LPO X PTM and DNA damaged sperms X PTM. A positive correlation (r = 0.069) was found among DNA damaged sperms and PTM during the present study, which had been earlier established in bovine (Kirk *et al.,* 2005). On the contrary, Mahmoud *et al.* (2015) and Badr *et al.* (2010) observed a significant negative correlation between DNA damaged sperms and motility in buffalo. It has been indicated that motility may be a relevant physiological marker for DNA intact spermatozoa. Irvine *et al.* (2000) also reported that sperm samples with low motility had higher rate of acrosome damage. In contrast to this, Morris *et al.* (2002) revealed that semen with higher sperm motility had higher rate of DNA damage. A significant moderate correlation (r = 0.234) was obtained between FDA spermatozoa and LPO during the present study. Bansal *et al.* (2014) observed a very high correlation (r = 0.88) between acrosome

Table 2: Pearson coefficient correlation among different sperm parameters of buffalo bull spermatozoa

Sperm parameter	HBP	PTM	PDA Spermatozoa	FDA Spermatozoa	DNA -D	AR Spermatozoa	LPO
HBP	1.00	0.075	0.028	0.056	0.258	0.010	0.009
PTM	—	1.00	0.245	0.00	0.069	0.018	0.009
PDA spermatozoa	—	—	1.00	0.068	0.00	0.015	0.001
FDA Sperms	—	—	—	1.00	0.019	0.105	0.234
DNA –D	—	—	—	—	1.00	0.047	0.133
AR Spermatozoa	—	—	—	—	—	1.0	0.00
LPO	—	—	—	—	—	—	1.00

HBP: Heparin binding proteins, PTM: Post thaw motility, PDA: partially damaged acrosome, FDA: Fully damaged acrosome, AR: Acrosome reacted, LPO: Lipid per oxidation, DNA-D: Spermatozoa with denatured DNA.

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damage and LPO, which was much higher than that observed in this study. Positive correlation between acrosome damage and LPO indicated that oxidation of polyunsaturated fatty acids in the membrane damaged the acrosome region. A significant high positive correlation (r = 0.258) between HBPs and DNA damage indicated that HBPs also protects double stranded DNA of buffalo bull spermatozoa.

Relationship of *in vitro* capacitation with HBP and sperm function tests: Thirty one bulls were divided into G-1 (> 40% AR) and G-II (≤ 40% AR). There was no difference in HBP concentration, PTM and DNA damaged sperms among the two groups (Table 3). Percentage of spermatozoa with partially, fully damaged and acrosome reacted spermatozoa was significantly ($p \leq 0.05$) higher in G- I (29.51 ± 1.02, 12.08 ± 0.88, 48.12 ± 1.92) as compared to G-II (25.09 ± 1.73, 9.90 ± 1.06, 36.25 ± 0.94). MDA level (µmoles / 10^9 spermatozoa) was non-significantly ($p \geq 0.05$) higher in G-II as compared to G-I. MDA has been used in biochemical assays to monitor the degree of per-oxidative damage in spermatozoa (Aitken and Fisher, 1994) and exhibited an excellent correlation with the degree to which sperm function is impaired in terms of motility and the capacity for sperm-oocyte fusion (Aitken *et al.,* 1993 and Sidhu *et al.,* 1998). Small amounts of ROS have been presented to be required for several functions of spermatozoa, but their excessive levels can negatively impact the quality of spermatozoa & impair the fertilizing capacity (Tvrda *et al.,* 2011). Therefore, oxidative stress affects the fluidity of sperm plasma membrane as well as integrity of DNA in the nucleus (Aitken 1999). Increase in membrane fluidity leads to sperm capacitation. Higher level of acrosome damage (cryocapacitation like changes) observed in frozen- thawed spermatozoa of G-I as compared to G-II may be due to premature capacitation during freezing. Spermatozoa itself produce small amounts of ROS that are essential to many of physiological process i.e. capacitation, hyperactivation and sperm oocyte fusion (Duaar *et al.,* 2000 and Aitken *et al.,* 2003). Low level of ROS has also been shown to be essential for fertilization, acrosome reaction and motility. Low level of MDA in G- I may be the reason for higher rate of acrosome reaction as compared to G-II. PTM, DNA integrity and HBP concentration did not vary among the two groups, which revealed non-dependence of capacitation and acrosome reaction on these sperm parameters.

Relationship of Conception rate with HBP and sperm function tests: Ten bulls (22-31) were divided into two groups; G-I (> 35 % conception rate) and G-II (< 35 % conception rate). Concentration of HBPs was significantly ($p \leq 0.05$) higher in G-I (2.03 ± 0.15 mg / 10^9 spermatozoa) as compared to G-II (1.31 ± 0.15

Table 3: Comparison in sperm parameters among the groups on the basis of rate of acrosome reaction

Sperm parameter	G-I	G-II
HBP (mg / 10^9 spermatozoa)	2.28 ± 0.12^a	2.15 ± 0.19^a
Motility (%)	58.79 ± 1.14^a	59.16 ± 0.99^a
PDA spermatozoa (%)	29.51 ± 1.02^a	25.09 ± 1.73^b
FDA spermatozoa (%)	12.08 ± 0.88^a	9.90 ± 1.06^b
DNA denatured Spermatozoa	23.61 ± 2.95^a	23.68 ± 2.05^a
MDA (µmoles / 10^9 Spermatozoa)	116.44 ± 15.90^a	145.24 ± 16.43^a
Acrosome reacted spermatozoa (%)	48.13 ± 1.92^a	36.25 ± 0.94^b

G-I: > 40 % acrosome reacted spermatozoa, G-II: ≤ 40 % acrosome reacted spermatozoa

Different superscripts (a and b) indicate the difference at 5% level of significance

PDA: Partially damaged acrosome, FDA: Fully damaged acrosome, MDA: Malondialdehyde

Table 4: Comparison in sperm parameters among the groups on the basis of conception rate

Sperm parameter	G-I	G-II
HBP (mg / 10^9 spermatozoa)	2.03 ± 0.15[a]	1.31 ± 0.047 [b]
Motility (%)	58.50 ± 0.61[a]	60.80 ± 2.26[a]
PDA spermatozoa (%)	20.46 ± 1.14[a]	18.00 ± 1.46[a]
FDA spermatozoa (%)	13.23 ± 1.23[a]	16.80 ± 1.62[a]
DNA denatured Spermatozoa	29.26 ± 3.99[a]	28.62 ± 2.91[a]
In vitro Acrosome reaction (%)	44.93 ± 2.19[a]	39.20 ± 2.08[b]
MDA (µmoles / 10^9 Sperms)	172.27 ± 12.63[a]	± 23.16[a]
Conception rate (%)	47.26 ± 3.51[a]	31.10 ± 2.03[a]

G-I: > 35 % acrosome reacted spermatozoa, G-II: ≤ 35 % acrosome reacted spermatozoa

Different superscripts (a and b) indicate the difference at 5% level of significance

PDA: Partially damaged acrosome, FDA: Fully damaged acrosome, MDA: Malondialdehyde

mg / 10 ^{9} spermatozoa, Table 4). It indicated that higher HBP concentration improves the fertility in buffalo. Singh *et al.* (2016) also revealed that buffalo bulls with higher fertility had higher concentration of HBP (0.46 ± 0.02 mg / 10^9 sperms) as compared to their counter parts (0.25 ± 0.01 mg / 10^9 sperms). Singh *et al.* (2014) postulated that HBP concentrations were drastically suppressed in buffalo bulls with sub-fertility. Percentage of acrosome reacted spermatozoa was also significantly ($P \leq 0.05$) higher in G-I (44.93 ± 2.19%) than G-II (39.20 ± 2.08). These observations reveal that higher concentration of HBP in high fertility bulls might also contribute toward higher capacitation status. In cattle bulls, Sprott *et al.* (2000) achieved 15-17% higher pregnancy rates in females inseminated with HBP positive spermatozoa than those inseminated with HBP-negative spermatozoa. It was postulated that poor reproductive performance in bulls could be partly due to lower levels of HBP (Harshan *et al.,* 2006). There was no difference in PTM, PDA-, FDA-, DNA damaged- sperms and LPO among the two groups (58.5 ± 0.61%, 20.46 ± 1.14%, 13.23 ± 1.23%, 29.26 ± 3.99%, 172.27 ± 2.19 µmoles / 10^9 spermatozoa vs 60.80 ± 2.26, 18.0 ± 1.46, 16.80 ± 1.62, 28.62 ± 2.91, 174.88 ± 23.16 µmoles / 10^9 spermatozoa). The results revealed that fertility of semen depends more upon HBP concentration and in vitro capacitation / acrosome reaction rather than acrosome damage, DNA integrity and LPO.

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