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Effect of Seminal Zinc, Calcium, Oxidative Stress and Protein Profile on Semen Quality of Crossbred Bulls

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ABSTRACT

The objective of present study was to investigate the effect of endogenous minerals (Zn and Ca), seminal proteins and oxidative stress on semen quality of crossbred bulls. Two crossbred bulls with history of good initial quality, high sperm motility percentage, and freezable ejaculates and poor initial quality, low sperm motility percentage, and donating mostly non-freezable ejaculates (Bull B), respectively were utilized. Six ejaculates from each bull were used and categorized into high progressive motile as good quality and low progressive motile as poor quality ejaculates groups. Total 24 ejaculates were taken during entire period of study. The level of Zn, Ca in seminal plasma and Ca in sperm pellets was found significantly ($P<0.05$) higher in good quality ejaculates of Bull A compared to poor quality ejaculates of Bull B; however, the level of reactive oxygen species and malondialdehyde was significantly higher ($P<0.05$) in poor quality ejaculates of Bull B compare to good quality ejaculate of Bull A. The 25 kDa protein band was prominent only in good quality ejaculate of Bull A. It was concluded that several proteinaceous antioxidant enzymes which may be present in 25 kDa band and minerals like Zn and Ca as a cofactors of these enzymes could be responsible for good quality semen ejaculates of Bull A.

Keywords: Zn, Ca, Oxidative stress, Antioxidant enzymes, Semen quality

There are several seminal factors like zinc, calcium (Singh *et al.,* 2018), presence of poor seminal protein (Kumar *et al.,* 2012) and oxidative stress in bulls (Simoes *et al.,* 2013) which affect the sperm motility. The role of various minerals present in seminal plasma in male reproduction is well established in different species. Zinc is a most important mineral found as a cofactor in more than 300 enzymes (Osredkar and Sustar, 2011) and have an important role in multiple biological processes including DNA, RNA, and protein synthesis (Al-Fartusie and Mohssan, 2017). Localization of zinc in the sperm middle piece in association with lipoprotein fraction suggests that it is involved in the catabolism of lipid, which is the principal source of energy required for the movement of spermatozoa (Singh *et al.,* 2018). It plays an important role in the prostate, epididymal and testicular functions (Ebisch *et al.,* 2006) and influences serum and seminal testosterone concentration. Zn has antioxidative properties and plays an important role in scavenging reactive oxygen species (Kerns *et al.,* 2018). Thus the reduced levels of zinc may result in poor sperm quality. Another mineral present in seminal plasma along with zinc is calcium which affects sperm motility. The influx of calcium regulates motility and triggers multiple physiological events in spermatozoa such as hyperactivation, chemotaxis, capacitation and acrosome reaction (Mannowetz *et al.,* 2013). Oxidative stress is another factor which determines the quality of semen at post thaw stage. Oxidative stress in male occurs due to imbalance between the oxidants and antioxidants (Agarwal and Prabakaran, 2005). Uncontrolled generation

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of Reactive oxygen species (ROS) due to oxidative stress causes lipid peroxidation of sperm plasma membrane. This may lead to mitochondrial disruption and chromatin fragmentation of sperm DNA resulting into increased number of dead, moribund and defective spermatozoa that may hamper sperm motility (Simoes *et al.,* 2013).

Several proteins are present in seminal plasma and spermatozoa which also have a positive relation with motility and fertility of spermatozoa. Various proteins such as clusterin, cholesterol-binding protein in stallion (Dacheux *et al.,* 2016), Heparin-binding proteins in mammal (Manjunath *et al.,* 2007), BSP protein homologs in seminal plasma of rams, PDC-109 in Buffalo and goat, spermadhesins in bulls, rams (Bergeron *et al.,* 2005), and bucks in the seminal plasma has been described. Several of these seminal plasma proteins found in the form of the antioxidant enzyme and as cofactors like zinc and calcium exert a cumulative effect on sperm motility, viability, freezability, sperm capacitation and fertilization (Asadpour *et al.,* 2007). Therefore, the present study was designed to investigate the role of the above mentioned seminal factors on semen quality of crossbred bulls.

MATERIALS AND METHODS

The proposed study was conducted at the Germ Plasm Centre (GPC), Division of Animal Reproduction, Indian Veterinary Research Institute (IVRI), Izatnagar, Bareilly (UP). The procedure involving animals handling and care were conducted as per the norms of Institutional Animal Ethics Committee. Two crossbred bulls A and B were kept in isomanagerial conditions. Out of the two, one bull had recorded history of good quality, high sperm motility percent and freezable ejaculates (Bull A) whereas other with a previous history of poor quality, low sperm motility percent and donating mostly non freezable ejaculates (Bull B). Ejaculates with progressive motility ≥70 % from Bull A and Bull B were categorized as good quality ejaculates and the ejaculates with progressive motility ≤50% from Bull A and Bull B were categorized as poor quality ejaculates. Six ejaculates from each bull were categorized into high motile and low motile groups. Thus, a total of 24 ejaculates were included in the experiment. Criteria of categorization into good and poor motile ejaculates was based on progressive motility. Physical and morphological characteristics like volume, color, concentration, consistency, mass and individual progressive motility were evaluated immediately after semen collection. After extending the semen with tris egg yolk extender, individual progressive motility of spermatozoa were counted in 4-5 randomly selected field under high power objective (40x) of phase contrast microscope after putting a cover slip over it. Sperm livability and abnormalities were estimated by using Eosin-Nigrosin stain as method described by Swanson and Bearden (1951). Ejaculates were centrifuged at 4000 rpm at 4ºC for 10 min for separation of seminal plasma and the cellular portion containing spermatozoa for estimation of seminal plasma protein profile through SDS PAGE. Separated seminal plasma and sperm pellets were preserved at -20ºC for further analysis.

Estimation of seminal zinc, calcium and seminal plasma protein profile

The Zn level in seminal plasma of individual semen ejaculate at fresh stage was estimated by atomic absorption spectrophotometry (AAS) method described by Smith *et al.* (1979). The calcium was estimated by kit method (Coral Clinical System, Lot: RCAL1052), as per the manufacturer's protocol. Seminal protein concentration was estimated by nanodrop technique; however, isolated and purified protein of each group was pooled and analyzed by discontinuous Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE) as described by Laemmli (1970). 20 µl of seminal plasma was loaded into the gel for electrophoresis.

Estimation of seminal oxidative stress

Reactive oxygen species were measured by Nitroblue tetrazolium blue (NBT) assay in sperm pellets as described by (Esfandiari *et al.,* 2003). Nitroblue tetrazolium (0.1%) was prepared in PBS by adding 10 mg of NBT powder (Sigma, cat. N.T-4000) to 100 mL of PBS (pH 7.2) and stirred at room temperature for 1 hour. The NBT solution was filtered using filter paper. The NBT staining was done for spermatozoa by adding equal volumes of 0.1% of NBT solution and incubated for 30 minutes at 37^0C. The smear was prepared from the pellet and air-dried. The air-dried smears were stained with Wright stain and a total of 100 spermatozoa were scored under 100X magnification. Spermatozoa in the smears were scored as: formazan occupying 50% or less of the cytoplasm and more than

50% of the cytoplasm. Spermatozoa cytoplasm occupied by more than 50 percent of formazan was assessed as positive for ROS. Lipid peroxidation level in seminal plasma was measured by determining the malondialdehyde (MDA) production, using thiobarbituric acid (TBA) as per the method of Buege and Aust (1978) and modified by Suleiman *et al.* (1996).

STATISTICAL ANALYSIS

Statistical analysis of different parameters was done by using the General Linear Model. Tukey test was used as a postdoc test. To find the association between different parameters Pearson correlation coefficient was used. The analysis was done by JMP software version 9.0.

RESULTS AND DISCUSSION

In the present study the ejaculates of Bull A were categorized into good quality ejaculates (Gr I), poor quality ejaculate (Gr II) and of Bull B as good quality ejaculate (Gr III) and poor quality ejaculate (Gr IV). The mass motility, initial progressive motility (Fig. 3B) and livability (Fig. 1A) of good quality ejaculate of Bull A were significantly (P<0.05) higher than poor quality ejaculate of Bull B (Table 1). The zinc level was found significantly (P<0.05) higher in good quality ejaculates of Bull A compared to poor quality ejaculates of Bull B which was in agreement with Tvrdá *et al*. (2013) (Table 1). The level of calcium in seminal plasma and sperm pellets was significantly (P<0.05) higher in good quality ejaculates of Bull A compared to poor quality ejaculates of Bull B which was in agreement with previous studies (Kadirvel, 2006; Rajoriya *et al.,* 2016; Beigi *et al.,* 2019). The level of ROS (Fig. 2A, B & 3C), MDA (Fig.3D) and sperm abnormalities (Fig. 1B & 3A) was significantly higher (P<0.05) in poor quality ejaculates of Bull B compare to good quality ejaculate of Bull A (Table 1). The seminal parameters like initial progressive motility, livability, zinc, calcium in seminal plasma and sperm pellet were significantly (P<0.05) higher in good quality ejaculates compared to poor quality ejaculates; however, the level of sperm abnormalities, ROS and MDA were significantly (P<0.05) higher in poor quality ejaculates compared to good quality ejaculates within the ejaculates of Bull A and Bull B (Table 1).

In the present study, protein concentration in seminal plasma of Bull A and Bull B was 106 and 95 mg/dl. The results of SDS PAGE revealed that there were 13 protein bands of 190, 83.5, 68, 57, 40, 33, 30, 27, 25, 22, 20, 15, and 10 kDa molecular weight in the ejaculates of all the four groups (good and poor ejaculates of Bull A and Bull B). All the bands were common in all four groups except 25 kDa (Fig. 4). The 25 kDa protein band was prominent in only good quality ejaculate of Bull A.

Table 1: Comparative result of different parameters in good and poor quality ejaculates of crossbred bull semen

Attributes	Bull A		Bull B		Overall	
	Gr I	Gr II	Gr III	Gr IV	Bull A	Bull B
Mass motility	3.83 ± 0.40^{a}	2.16 ± 0.31^{bc}	3.00 ± 0.00^{ab}	1.50 ± 0.22^{c}	3.00 ± 0.35^{A}	2.25 ± 0.25^{B}
IPM (%)	76.66 ± 2.11^{a}	43.33 ± 3.33^{b}	71.66 ± 1.67^{a}	45.00 ± 2.24^{b}	60.00 ± 5.37^{A}	58.33 ± 4.23^{A}
Conc^{n} (million/ml)	1237.67 ± 208.15^{a}	1067.50 ± 112.40^{a}	721.50 ± 107.51^{ab}	464.83 ± 58.61^{b}	1152.58 ± 115.66^{A}	593.16 ± 70.04^{B}
Livability (%)	84.99 ± 3.21^{a}	51.39 ± 4.35^{b}	84.85 ± 2.24^{a}	56.42 ± 2.83^{b}	68.19 ± 5.68^{A}	70.63 ± 4.62^{A}
Abnormality (%)	6.25 ± 0.19^{c}	7.83 ± 0.25^{b}	8.18 ± 0.15^{b}	10.51 ± 0.19^{a}	7.046 ± 0.28^{B}	9.34 ± 0.37^{A}
Volume(ml)	3.83 ± 0.29^{a}	4.18 ± 0.66^{a}	4.16 ± 0.31^{a}	5.60 ± 1.04^{a}	4.00 ± 0.35^{A}	4.88 ± 0.56^{A}
ROS (%)	26.38 ± 2.06^{b}	71.06 ± 3.42^{a}	27.95 ± 1.46^{b}	78.38 ± 2.57^{a}	48.72 ± 6.99^{A}	53.17 ± 7.73^{A}
MDA (µmol/10µl)	0.24 ± 0.01^{c}	0.326 ± 0.02^{b}	0.291 ± 0.02^{bc}	0.439 ± 0.01^{a}	0.283 ± 0.02^{B}	0.365 ± 0.02^{A}
Zinc (ppm)	2.31 ± 0.21^{a}	0.67 ± 0.04^{b}	1.90 ± 0.15^{a}	0.50 ± 0.06^{b}	1.489 ± 0.27^{A}	1.2028 ± 0.23^{B}
Ca plasma (mg/dl)	34.67 ± 0.46^{a}	33.00 ± 1.61^{b}	23.29 ± 2.56^{a}	21.55 ± 2.78^{b}	33.84 ± 0.84^{A}	22.42 ± 1.82^{B}
Ca pellet (mg/100 million)	52.97 ± 0.86^{a}	44.78 ± 0.83^{b}	42.79 ± 0.87^{b}	39.10 ± 0.83^{c}	48.88 ± 1.36^{A}	40.95 ± 0.80^{B}

Means bearing different superscripts (a, b, c) (A, B) in a row and column, respectively differ significantly (P<.05); IPM = Initial progressive motility, Conc = Concentration, ROS = Reactive oxygen species, MDA = Malondialdehyde, Ca plasma = calcium in seminal plasma, Ca pellet = Calcium in sperm pellet.

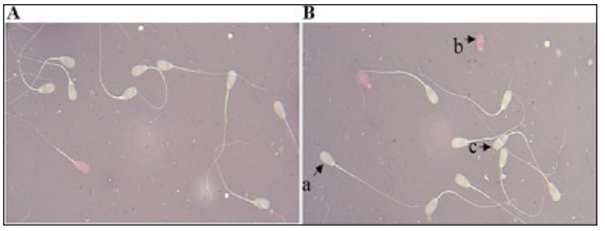

Fig. 1: Assessment of sperm viability and abnormality of crossbred bull spermatozoa using eosin-nigrosine stain, **(A)** live (unstained) and dead spermatozoa (stained) (100x) and **(B)** Abnormal spermatozoa (100x); a-Normal; b-Free head; c- Bent tail.

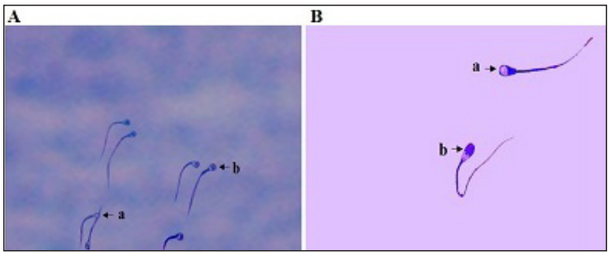

Fig. 2: Assessment of ROS level in crossbred bull spermatozoa using nitrotetrazoleum blue stain, A (40x) and B (100x); a-Formazan occupied <50 cytoplasm showing good quality of spermatozoa; b- Formazan occupied >50 cytoplasm showing poor quality of spermatozoa

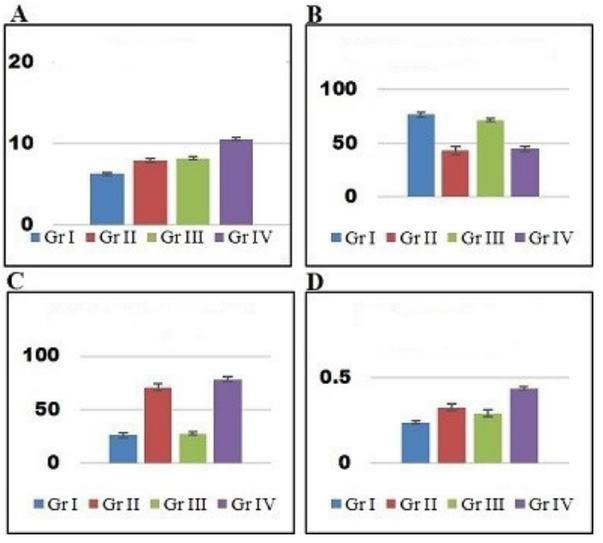

Fig. 3: Graphs showing relative levels of different parameters in GrI, GrII, GrIII and GrIV respectively; **(A)** Abnormality; **(B)** Initial progressive motility; **(C)** ROS in sperm pellets and **(D)** MDA concentration in seminal plasma

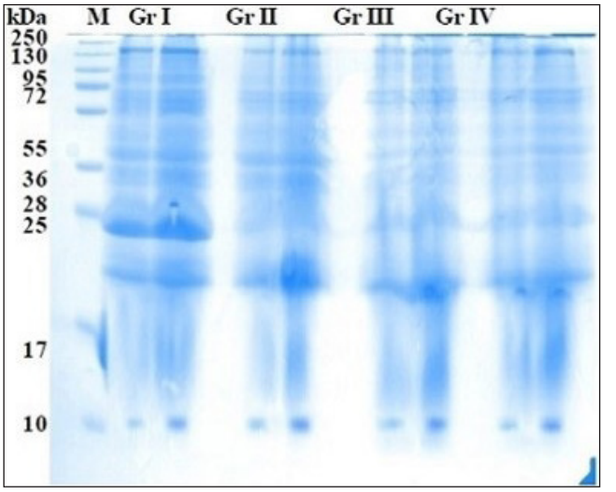

Fig. 4: Electrophoresis pattern showing seminal plasma protein profile of GrI, GrII, GrIII and GrIV ejaculates respectively

Results indicate that there is a mutual and deep association between the minerals, oxidative stress and proteins of the seminal plasma and they all exert a cumulative effect on sperm motility. Higher seminal zinc level in good quality ejaculates of Bull A indicates that zinc is necessary for maintaining the stability of sperm chromatin and plasma membrane; inhibit apoptosis for normal sperm morphology during spermatogenesis (Zhao *et al.,* 2016). Zinc is the cofactor of several enzymes like superoxide dismutase, sorbitol dehydrogenase, lactate dehydrogenase, alkaline phosphatase (Singh *et al.,* 2018) and plays an important role in the synthesis of metallothioneins (low-molecular-weight Zn-binding proteins) which have the antioxidant property for protecting biological tissues from oxidative damage (Zhao *et al.,* 2016). Due to reduced zinc concentration in the poor quality ejaculates of Bull B, these above antioxidant enzymes can be dysfunctional. The impaired antioxidant system might have resulted in higher ROS and MDA in the poor quality ejaculates of Bull B. The elevated levels of above mentioned parameters may be one of the factors responsible for higher dead or defective sperm count in the poor quality ejaculates as LPO results in lethal structural alterations in sperm plasma membrane.

The higher level of seminal calcium facilitates the influx of Ca^{+2} from extracellular to intracellular environment of sperm which is required to initiate the capacitation and acrosomal reaction and ultimately for gaining motility

and sperm-egg interaction (Beigi *et al.,* 2019). Higher intracellular calcium indicates that there could be higher number of calmodulin (Intracellular calcium receptor), Na/K ATPase, inositol and 1, 4, 5 triphosphatase receptor (Intracellular calcium store receptor) (Costa *et al.,* 2010). Calmodulin also modulates the protein secretion and upon binding to calcium, it activates different enzymes especially protein kinases, phosphatases and phosphodiesterases (Darszon *et al.,* 2011). Protein kinases are responsible for the phosphorylation of the sperm proteins due to that the spermatozoa gain its motility (Beigi *et al.,* 2019).

Higher level of minerals like zinc and calcium was found in good quality semen ejaculates of Bull A compared to poor quality ejaculate of Bull B and these minerals are the cofactors of the various enzymes. The seminal plasma proteins primarily secreted from the epididymis, cover the sperm membrane, form the sperm surface proteins and these above mentioned antioxidant enzymes and ion channel proteins in the sperm membrane mainly comes from seminal plasma proteins, that play various roles in sperm function, sperm-oocyte interaction and fertilization. The SDS PAGE analysis revealed that protein band of 25 kDa was positively correlated with progressive motility. There could be several proteins present in the 25 kDa protein band including above mentioned enzymes which facilitate and support the viability, progressive motility of spermatozoa. Thus we can observe a close relationship between minerals like zinc and calcium with seminal plasma proteins and their role in antioxidant property based on above mentioned findings. In future mass spectrophotometry can reveal the possibility of the presence of individual proteins that will help to identify the role of these proteins in facilitating the progressive motility of the spermatozoa.

CONCLUSION

It was concluded from the present study that several proteinaceous antioxidant enzymes and Zn and Ca as a cofactors may be present in 25 kDa band. Their presence may be one of the factors responsible for good quality semen ejaculates of Bull A in comparison to poor quality ejaculates of Bull B.

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CONFLICT OF INTEREST

The authors declare that they do not have any conflict of interest.

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