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Qualitative and Quantitative Estimation of Osteopontin in Bull Semen and its Effect on Sperm Quality Parameters and Fertility Rate *In vivo*

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ABSTRACT

Background: Osteopontin plays a crucial role in fertilization and subsequent embryonic development.

Methods: The fresh semen samples were collected from crossbred Jersey and Haryana breeding bulls. The proteins in the seminal plasma and sperm membrane were precipitated and analysed by SDS-PAGE followed by western blotting. Based on the concentration of osteopontin in the seminal plasma, the bulls were grouped as high (≥20 pg/ml), medium (10-20 pg/ml) and low (≤10 pg/ml) OPN groups.

Result: Total 13 and 14 different proteins were identified in the seminal plasma and sperm membrane, respectively. The sperm velocity parameters (straight line velocity and straightness) and functional membrane integrity showed significant difference among high, medium and low OPN groups. Significant difference (P<0.05) was observed in malondialdehyde, SOD and glutathione reductase levels. Bulls which are having high osteopontin concentration in the semen had less sperm abnormality, less apoptotic cells, better resistance to cryopreservation, less damaged chromatin and higher conception rate (64.80±1.09) than bulls having low osteopontin concentration in their semen. Therefore, osteopontin may be a valuable protein marker to predict fertility in bulls.

Key words: Bull, Fertility, Osteopontin, Semen, Sperm.

INTRODUCTION

In India, it was reported that approximately 20% of the bulls are sub fertile in natural condition (Mukhopadhyay *et al*., 2010). Sperm morphological measurements are not always indicative of actual fertility as 20-25% difference in conception rate was reported (Larson and Miller, 2000). Therefore, accurate and predictive protein markers need to be identified for selection of breeding bulls (Chacur, 2012). The spermatozoa are transcriptionally inactive. Hence, the only comprehensive method to understand the molecular function in spermatozoa is via proteomics (Pixton *et al*., 2004). The proteins of seminal plasma have activities in anti-apoptosis and cell survival that promotes capacitation of sperm cells by increasing the number of heparin binding sites on the sperm surface (Yathish *et al*., 2018). Osteopontin, a 55 kDa protein secreted from ampulla and seminal vesicle of bulls has been positively correlated with fertility and play an integral part in number of signal transduction pathways. With this preface, the experiment was designed to quantify osteopontin in semen and to compare the sperm quality parameters and *in vivo* fertility among bulls.

MATERIALS AND METHODS

Freshly ejaculated semen samples were obtained from 21 (12 Crossbred Jersey and 9 Haryana) bulls aged between 2 to 6 years maintained by Frozen Semen Bank, Khapuria, Cuttack, Government of Odisha. The bulls were given the following identification numbers by the bull station as C 235, C 254, C 261, C 268, C 273, C 274, C 285, C 291 C 294, C 302, C 307, C 332, H 617, H 624, H 649, H 656, H 666, H 678, H 694, H761 and H 920.

The freshly ejaculated semen was evaluated for sperm concentration and motility. The seminal plasma protein/ sperm membrane protein was separated as per standard

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procedure. These proteins were denatured and were separated by SDS PAGE. Pre-stained standard marker proteins (Broad range marker 7.1-209 kDa, Bio-Rad, India) and Precision plus protein standards (10 -250 kDa, Bio-Rad, India) were used to determine the location of protein of interest based on its molecular weight. The gel was stained with coomassie brilliant blue R- 250 (0.15%) followed by destaining in a mixture of methanol (25%) and acetic acid (10%) in distilled water. The gel was visualized in Gel Documentation and Analysis System (Gel-Doc. Bio- Rad, UK) and stored in acetic acid (7%). Western blotting (Electro-blotting) was carried out using Trans blot SD cell (Bio-Rad) as per the standard procedure with goat anti rabbit horseradish peroxidase as conjugate for identification of osteopontin (OPN) band.

The quantification of osteopontin was carried out using commercial ELISA Kit. Based on the quantity of OPN present in the seminal plasma, bulls were categorized into three groups as high OPN (>20 pg/ml), Medium OPN (10-20 pg/ml) and low OPN (<10 pg/ml) groups. Bulls included in the high OPN groups (n=5) were C 302, H 666, H 649, H 617 and H 678. In medium OPN group (n=11), included bulls were C 261, C 268, C 274, C 273, C 254, C 285, C 307, H 920, H 624, H 761 and H 694. Bulls in low OPN group (n=5), were C 332, C 235, C 291, C 294 and H 656.

Sperm morphology, functional membrane integrity, acrosomal integrity and DNA integrity were studied in both fresh and frozen semen of all the bulls. The motility, sperm cell apoptosis and lipid peroxidation were studied in thawed semen from frozen semen straws. Parameters like catalase, superoxide dismutase and glutathione reductase were studied from the sperm cell lysate prepared from the thawed semen of frozen semen straws. Annexin-V-FITC apoptosis detection kit (Sigma - Aldrich, Saint Louis, USA) was used to detect the translocation of membrane phospholipid phosphatidylserine (PS). Lipid peroxidation level of spermatozoa was estimated by measuring the malondialdehyde (MDA) production, using thiobarbituric acid (TBA). The absorbance was measured at 535 nm under UV spectrophotometer (Cecil CE 2021, 2000 series). The MDA concentration was determined by the specific absorbance coefficient (1.56×10^5/molcm-3).

The field fertility data was obtained from the above mentioned bulls on non-return basis and was compared among them. All statistical analyses were carried out using the Statistical Package for Social Sciences programme (SPSS), version 20.00 software for windows (SPSS Inc. Chicago, IL, USA).

RESULTS AND DISCUSSION

The seminal plasma protein of the bulls under study showed 13 different bands of different molecular weight ranging from 8.5 to 205 kDa in twelve crossbred Jersey and nine Haryana bulls. Similarly, the sperm membrane protein of the bulls under study showed 14 different bands of different molecular weight ranging from 8.5 to 205 kDa in twelve crossbred Jersey and nine Haryana bulls. Among all protein bands present in seminal plasma and sperm membrane, only four bands having molecular weight 55 kDa (Osteopontin), 30 kDa (Fertility associated antigen - FAA), 26 kDa (Lipocalin-type PGD synthase - LPGDs) and 24 kDa (Type-2 tissue inhibitor of metalloproteinases - TIMP-2) were considered as the Fertility Associated Proteins (Fig 1). Similarly, 3 protein bands of molecular weight 14, 15 and 30 kDa were found in bovine bull seminal plasma (Kadoom *et al*., 2016) and 13 protein bands ranging from 6.5 kDa to 204 kDa in the seminal plasma of Haryana bulls were recorded (Dixit *et al*., 2016) out of which the presence of 5 protein bands (8.5, 26, 43, 66 and 160 kDa) corroborates with the present findings. The variation in the number of protein bands observed in the present study might be due to the inherent character of species difference between cattle and buffaloes or it may be due to methodology and media involved in protein isolation (Arangasamy *et al*., 2005).

In the present study, 14 bands of different molecular weight were recorded in the sperm membrane protein. Out of the protein bands, only 15/14 kDa was present in all the twelve crossbred Jersey and nine Haryana bulls. The presence of 14 protein bands varying from 16-205 kDa in Bhadawari buffalo bulls and 17 protein bands ranging from

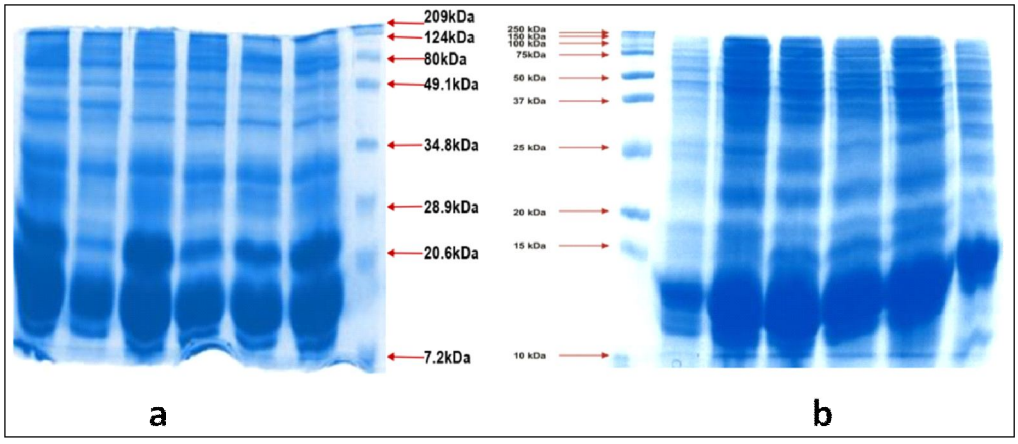

Fig 1: SDS PAGE showing bands of fertility associated proteins present in seminal plasma (a) and sperm membrane (b).

6.5-174 kDa in Haryana bulls (Dixit *et al*., 2016) were reported. Earlier studies confer the relationship of sperm membrane proteins with fertility indicating that some of these proteins are potential biomarkers of the male reproductive status and the difference in protein profile could be due to species specific variation (Dixit *et al*., 2016).

Four fertility associated proteins with molecular weight ranging from 24 kDa to 55 kDa were observed in seminal plasma and sperm membrane proteins in the present study. Using two-dimensional (2-D) gel electrophoresis, 2 seminal plasma proteins (26 and 55 kDa) were identified with high fertility and 2 proteins (16 and 16 kDa) were correlated with low fertility (Killian *et al*., 1993). The 55-kDa fertility-associated protein was identified as osteopontin (Cancel *et al*., 1999) and the 26-kDa fertility-associated protein was identified as lipocalin-type prostaglandin D synthase (Gerena *et al*., 1998). In the present study, the protein band of molecular weight 55 kDa, identified as Osteopontin was present in seminal plasma of 18 bulls (85.711%) and sperm membrane of 14 bulls (66.66%). A major heparin binding protein of molecular weight ranging from 28-30 kDa known as fertility associated antigen was present in the seminal plasma of 18 bulls (85.711%) and sperm membrane of 14 bulls (66.66%).

Western blotting analysis for osteopontin and its quantification

Seminal plasma and sperm membrane proteins were subjected to single dimensional SDS PAGE and western blot analysis (Fig 2). The OPN antibody recognized three immune reactive bands of molecular weight 55 kDa, 40 kDa and 22 kDa in the seminal plasma protein. All the bands have different intensities as viewed on nitrocellulose membrane (Fig 2). Solubilised sperm membrane proteins were also subjected to western blot analysis but no bands were visible in nitrocellulose membrane. The first report of OPN in bovine seminal plasma was on the basis of N- terminal amino acid sequencing comparisons, western blot analysis with OPN specific antisera and carbohydrate and phosphorylation analysis (Cancel *et al*., 1997). The OPN in seminal plasma of Holstein bulls was detected and its positive correlation with fertility was established (Cancel *et al*., 1999).

Two immunorecative bands (55 and 25 kDa) were in cauda epididymal fluid and testicular parenchyma (Erikson *et al*., 2007). Additional immunoreactive bands at 60, 40 and 22 kDa in testicular parenchyma and 45 kDa in cauda epididymal fluid were recorded. The multiple bands observed on nitrocellulose membrane may be due to use of polyclonal antibody against the whole seminal plasma which enabled the detection of putative immunogenic contaminants or there may be multiple isoforms of osteopontin present in seminal plasma (Cancel *et al*., 1997). The levels of osteopontin in seminal plasma of different bulls were estimated. The highest and lowest concentrations of osteopontin recorded in the seminal plasma of bulls were 34.88 pg/ml and 2.9 pg/ml. The bulls showing more than 20 pg/ml, 10 - 20 pg/ml and less than 10 pg/ml of OPN were categorized into high OPN, medium OPN and low OPN group, respectively in the present study.

Sperm quality parameter analysis

Sperm morphology in fresh and thawed frozen semen samples were assessed in high, low and medium osteopontin (OPN) group bulls. The percentage of major, minor and total sperm abnormalities in fresh and frozen semen were estimated. The percentage of sperm abnormality was not significantly different between the groups. However, the total sperm abnormality was low in high OPN group as compared to other groups.

Motility and velocity parameters of the sperm cells (Fig 3) were assessed in thawed frozen bull semen sample of high, medium and low OPN groups. Progressive forward motility, non-progressive motility, total motility, static cells, curvilinear velocity, average path velocity, linearity, wobble, amplitude of lateral head displacement and beat cross frequency did not differ significantly between the high, medium and low OPN groups but the straightness differ significantly between ($p<0.05$) high and low OPN groups. A significant difference ($p<0.05$) in straight line velocity was observed between medium and low OPN groups.

Functional membrane integrity was assessed by hypo osmotic swelling test (HOST) in fresh and thawed frozen semen of high, medium and low OPN bull groups (Table 1). The proportion of spermatozoa showing positive reaction to hypo osmotic swelling test was more in high OPN group bulls as compared to low OPN group bulls in fresh semen without any statistical significant difference. The number of HOST reacted sperm cells were significantly more ($p<0.05$)

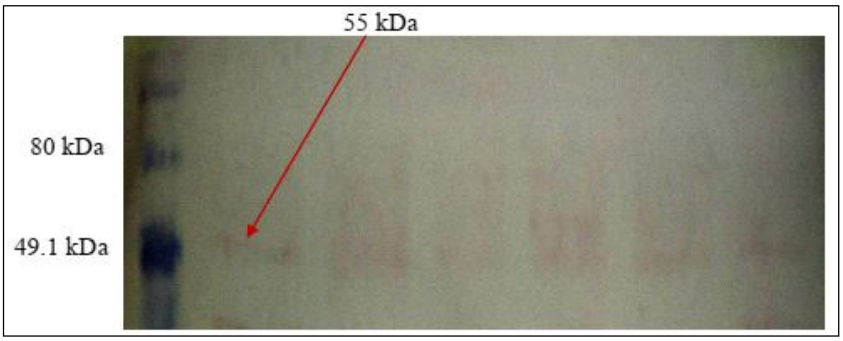

Fig 2: Representative western blot analysis of osteopontin in seminal plasma of crossbred Jersey and Haryana bulls.

Table 1: Mean (±S.E) sperm DNA integrity, functional membrane integrity (HOST) and acrosomal integrity in fresh and thawed frozen semen of high, medium and low OPN groups.

Parameters	Fresh semen			Thawed frozen semen		
	High OPN	Medium OPN	Low OPN	High OPN	Medium OPN	Low OPN
HOST (%)^{NS}	78.80±2.47	77.91±1.32	76.60±1.60	62.60±1.53^{a}	57.91±1.08^{b}	52.60±1.32^{c}
Acrosomal integrity (%)^{NS}	91.20±0.86	89.09±0.82	87.60±1.50	80.60±2.37	76.45±2.18	73.00±2.51
DNA integrity (%)*	96.40±0.51^{a}	95.55±0.39^{ab}	94.20±0.58^{b}	95.80±0.58^{a}	94.64±0.47^{a}	92.80±0.58^{b}

Means with different superscripts differ significantly ($p<0.05$).

Table 2: Mean (±S.E) Malondialdehyde (MDA) and antioxidative enzymes in thawed frozen semen of high, medium and low OPN groups.

Parameters	High OPN	Medium OPN	Low OPN
MDA (μ mol/ml)*	1.57±0.06^{a}	1.75±0.05^{a}	2.02±0.01^{b}
Catalase (U/ml)*	4.80±0.49^{a}	3.00±0.33^{b}	2.40±0.24^{b}
SOD (U/ml)*	7.80±1.15^{a}	4.91±1.09^{ab}	3.60±0.67^{b}
GR (U/ml)*	0.015±0.002^{a}	0.009±0.001^{ab}	0.007±0.001^{b}

Means with different superscripts differ significantly ($p<0.05$).

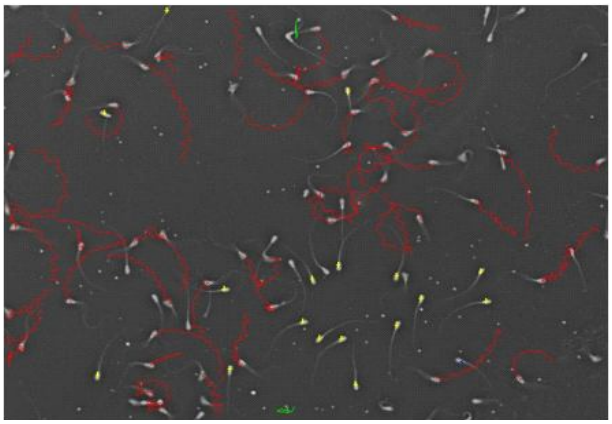

Fig 3: Photomicrograph of sperm motility (CASA, 600X).

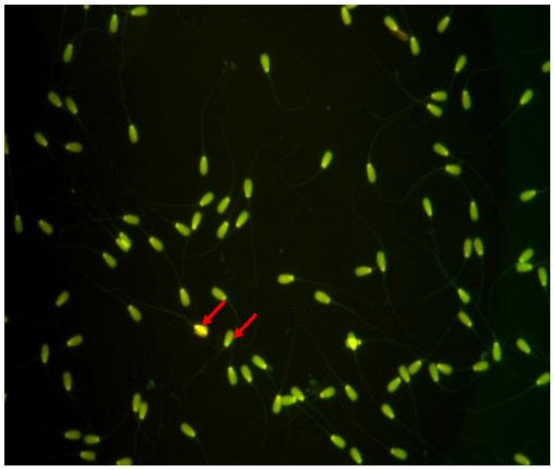

Fig 4: Photomicrograph of sperm showing intact and damaged chromatin (Acridine Orange, 600X) Green - ds DNA, Red - ss DNA.

in high OPN group as compared to medium OPN group bulls in frozen semen. Similarly, the HOST reacted sperm cells were highly significant ($p<0.01$) in high OPN group bulls as compared to low OPN group bulls. Acrosomal integrity of the sperm cells was assessed by Giemsa staining in fresh and thawed frozen semen of high, medium and low OPN bull groups (Table 1). Any significant difference in acrosomal integrity was not observed in any of the group of fresh and thawed frozen semen. The percentage of sperm cells with intact DNA was assessed (Fig 4) by acridine orange staining in both fresh and thawed frozen semen samples of high, medium and low OPN group bulls (Table 1). No significant difference was observed among three groups of fresh semen. But percentage of intact DNA of low OPN group was significantly ($p<0.05$) differed from other two groups of frozen semen.

The Malondialdehyde (MDA), catalase, superoxide dismutase and glutathione reductase level in thawed frozen semen samples of high, medium and low OPN group bulls was calculated (Table 2). Comparison of malondialdehyde level between low and medium OPN group bulls showed a significant ($p<0.05$) higher value in low OPN group bulls as compared to medium OPN group bulls. The MDA level between high and low OPN group bulls showed a highly significant ($p<0.01$) difference between the groups. The amount of catalase was significantly ($p<0.01$) higher in thawed frozen semen samples of high OPN group bulls as compared to medium and low OPN group bulls. The level of superoxide dismutase was significantly higher ($p<0.05$) in high OPN group bulls as compared to low OPN group bulls. The value of glutathione reductase was significantly ($p<0.05$) higher in high OPN group bulls as compared to low OPN group bulls.

The percentage of apoptotic sperm cells, viable sperm cells and necrotic sperm cells in the thawed frozen semen samples of high, medium and low OPN group bulls were estimated (Fig 5 and Table 3). The high OPN group bulls had a significantly ($p<0.05$) lower percent of apoptotic cells than low OPN group bulls. The high OPN group bulls had a significantly ($p<0.05$) higher percentage of viable sperm cells than low OPN group bulls. Any significant difference of necrotic sperm cells could not be observed in any of the group of bulls.

In vivo fertility study

The conception rate of high, medium and low OPN group bulls was 64.80±1.09, 57.36±1.38 and 42.54±3.65, respectively. The conception rate was highly significant

Table 3: Mean (±S.E) apoptotic, live and necrotic sperm cells of post thawed frozen semen in high, medium and low OPN groups.

Parameters	High OPN	Medium OPN	Low OPN
Apoptotic sperm cell*	9.20±1.02^{a}	12.18±0.95^{ab}	13.60±1.32^{b}
Live sperm cell*	56.60±1.40^{a}	52.55±1.18^{ab}	48.40±1.72^{b}
Necrotic sperm cell	34.00±1.41	35.09±1.51	38.00±1.09

Means with different superscripts differ significantly ($p<0.05$).

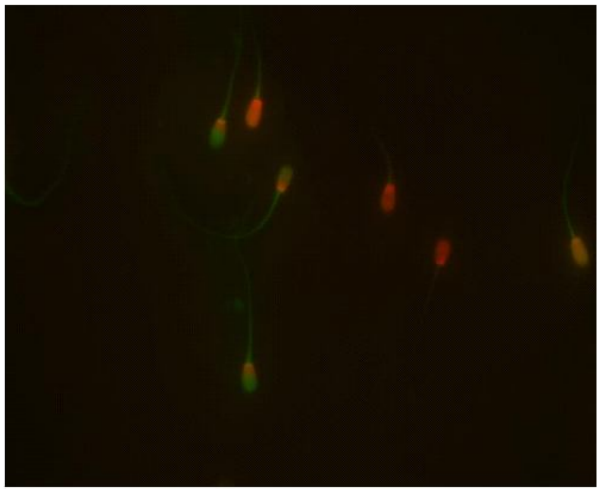

Fig 5: Photomicrograph showing live, apoptic and necrotic sperm cells (Annexin-V FITC, 600X).

($p<0.01$) in high OPN group bulls as compared to medium and low OPN group bulls and it showed significant ($p<0.05$) difference between medium and low OPN group bulls.

CONCLUSION

The protein profile of seminal plasma and sperm membrane showed variation in breeding bulls. The fertility associated proteins were expressed differentially in seminal plasma and sperm membrane protein in different bulls. Bulls which are having high osteopontin in their seminal plasma had better resistance to cryopreservation with less sperm abnormality, damaged chromatin in sperm cells, apoptotic sperm cells and more sperm cells with functional membrane integrity and higher conception rate. Therefore, osteopontin may act as a valuable marker to predict fertility in bulls.

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