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Ameliorative effects of osteopontin on sperm morphology of frozen thawed buffalo semen treated with sodium nitroprusside

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Abstract
The present experiment was conducted to study the ameliorative effects of exogenous supplementation of osteopontin (ONP) on sperm morphology of frozen thawed buffalo semen treated with sodium nitroprusside (SNP), a nitric oxide (NO) donor. Buffalo semen straws from 8 ejaculates in 6 bulls were procured from Central Frozen Semen Production and Training Institute, Hessarghatta, Banglore and stored at Semen Bank, Madras Veterinary College, Chennai. The frozen straws were thawed and seminal plasma and semen extender were removed from spermatozoa by centrifugation. Spermatozoa were suspended in 1mL capacitation medium (control), addition of 100µg/mL of OPN (treatment I), 100µM/mL of SNP (treatment II) and 100µg/mL of OPN + 100µM/mL of SNP (treatment III). Semen with capacitation medium without any treatment served as control. The contents were incubated at 37ºC for 4 h and the sperm morphology was determined by Rose Bengal stain technique. No significant difference was observed in morphologically normal spermatozoa between control (81.75% ± 0.16) and treatment I (83.85% ± 0.20). Among treatment groups, significantly (P<0.05) more morphologically normal spermatozoa were observed in treatment I as compared to treatment II (57.05% ± 0.07) and III (68.50% ± 0.09). On comparison with control, significantly (P<0.05) lesser number of morphologically normal spermatozoa were found in treatment II and III. Morphologically normal spermatozoa in treatment III were significantly (P<0.05) higher than treatment II. The results of this study revealed that SNP supplementation alone have detrimental effects on the sperm morphology and co-incubation with OPN partially ameliorated the toxic effects of SNP on the sperm morphology of frozen thawed buffalo semen.

Keywords: Osteopontin, sodium nitroprusside, sperm morphology, buffalo semen

Introduction
The role of seminal plasma proteins in the regulation of sperm functions is highly complex and several studies provided direct evidence that seminal plasma proteins were associated with the fertilizing capacity of sperm. While most of these proteins were found in the seminal plasma, some were also identified in sperm membrane. The seminal plasma, a complex mixture of secretions from testis, epididymis and accessory sex glands have factors/ proteins that modulate the fertilizing ability of sperm [1, 2, 3, 4, 5]. Proteins such as osteopontin (OPN), prostaglandin D synthase, bovine seminal plasma proteins (BSP A1, A2 and A3), heparin binding proteins (HBPs), fertility associated antigen (FAA), phospholipase A2, sperm adhesion Z13, clusterin and heat shock proteins (HSPs) have been reported as indicators of fertility [6, 7, 8, 9, 10]. The addition of seminal plasma to frozen thawed ram sperm improved motility, viability and mitochondrial respiration [11, 37]. Addition of seminal plasma also increased the resistance to spermatozoa of bull [13], ram [14] or red deer [15] to cryo-injury.
Oxidative stress (OS) is a promising field in sperm physiology. OS can be defined as the imbalance between pro-oxidative and anti-oxidative molecules in a biological system which arises as a consequence of excessive production of free radicals and impaired antioxidant defense mechanism [16, 17, 18]. Free radicals derived from oxygen are called reactive oxygen species (ROS), which include superoxide (O_2^-), hydrogen peroxide (H_2O_2), peroxyl (ROO^-) and hydroxyl (OH) radicals [19]. Those derived from nitrogen are called reactive nitrogen species (RNS). RNS include nitric oxide (NO^-), nitrogen dioxide (NO_2) and peroxynitrite anion ($ONOO^-$) [20]. RNS are often considered to be subclass of ROS [21]. Among RNS, NO is as an intercellular and intracellular messenger molecule controlling many physiological processes.

Low concentration of NO increased the motility and viability of spermatozoa. However, high concentration of NO decreased the sperm motility and viability in ram [22].

OPN is induced by OS and it exerts anti-oxidant effects by down regulating cellular hydrogen peroxide levels and by suppressing transcription of inducible nitric oxide synthase (iNOS) on various cells [23]. OPN genes can be used as a reference for the selection of superior quality bulls [24]. OPN gene is expressed in spermatozoa of ejaculated bull semen. Expression levels of OPN transcripts vary among the bulls. Correlation is found between expression levels of OPN gene transcripts and *in vitro* sperm characteristics [25]. Hence, the study was conducted to explore the ameliorative effects of exogenous supplementation of osteopontin (OPN) on sperm morphology of frozen thawed buffalo semen treated with sodium nitroprusside (SNP), a nitric oxide (NO) donor.

Materials and Methods

Materials

All the plasticware used for this study *viz*., centrifuge tubes, microcentrifuge tubes, microtips (different graduations) were purchased from Tarson India. All the glassware used in this study *viz*., laboratory bottles, microscope cover slips 18mm × 18mm, microscopic slides, conical flasks and beakers were purchased from Borosil, India. All the laboratory chemicals used in this study were procured from Sigma-Aldrich chemicals Co., USA.

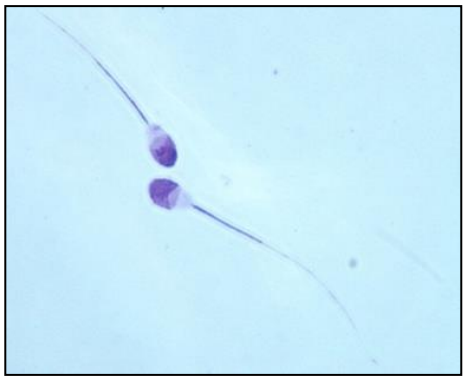
Methods

Sperm treatment

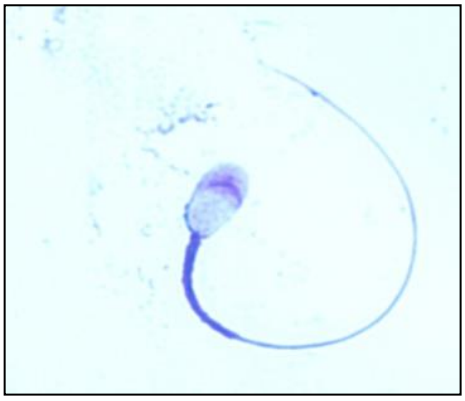
Buffalo semen straws from 8 ejaculates in 6 bulls were procured from Central frozen Semen Production and Training Institute, Hessarghatta, Banglore-51. The straws were collected in liquid nitrogen (LN_2-196 ºC) container, transported and stored in the Semen Bank, Madras Veterinary College, Chennai- 600 007.The frozen straws were thawed at 37ºC for 30 sec. and transferred to a test tube containing 5 ml of sperm capacitation medium (Table 1) and centrifuged at 50 g for 10 min. Supernatant was discarded and sperm pellet was reconstituted with 5 ml of fresh sperm capacitation medium and centrifuged as the same rate. Again, the supernatant was discarded and the sperm pellet was finally reconstituted in 1 ml of sperm capacitation medium (Control); supplemented with 100µg/ml OPN alone (Treatment I); 100µM/ml SNP alone (Treatment II); 100µg/ml OPN and 100µM/ml SNP (Treatment III). Sperm sample was incubated at 38±1˚C and 5% CO_2 in humidified air for 4 h. After incubation, the sperm morphology was examined from the above groups as described below.

Evaluation of sperm abnormality

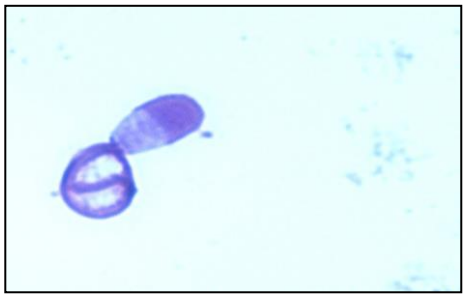
Sperm morphology was assessed by Rose Bengal stain (Figure 1A - 1 E). 250µl semen was emptied into a separate eppendorf tube containing 500µl of Tris buffer and then 3 drops of Rose Bengal stain was added. The contents were centrifuged at 2000-3000 rpm for 3 min. Then 1 mL Tris buffer was added. Again the contents were centrifuged at 2000-3000 rpm for 3 min. Supernatant was removed. Finally 100µl Tris buffer was added. One drop of the well mixed sample was placed on a clean grease free glass slide and covered with cover slip. Spermatozoa were observed under 40X. Spermatozoa showing head, mid-piece and tail abnormalities were included in total abnormalities. A minimum of 200 spermatozoa were observed [26].



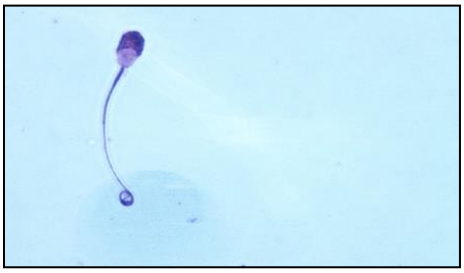
1A: Normal sperm



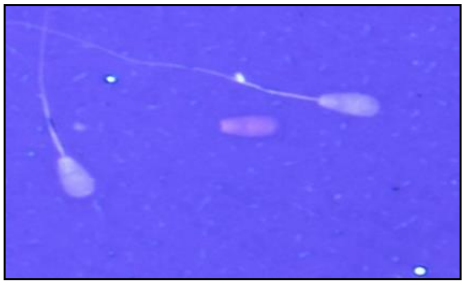
1B: Sperm with bent tail



1C: Sperm with dag defect



1D: Sperm with coiled tail



1E: Sperm with decapitated head

Fig 1: Sperm Morphology

Statistical analysis
Data were fed in Microsoft Excel and statistical analyses were conducted using SPSS for Windows 23.0 (IBM Corp.).Statistical design was carried out by completely randomized design (CRD). All the data were analyzed by one way analysis of variance followed by Duncan's multiple comparison test.

Results
Table 1 indicates that there was no significant difference in morphologically normal spermatozoa between control (81.75% ± 0.16) and treatment I (83.85% ± 0.20). Among treatment groups, significantly (P<0.05) more morphologically normal spermatozoa were observed in treatment I as compared to treatment II (57.05% ± 0.07) and III (68.50% ± 0.09). On comparison with control, significantly (P<0.05) lesser number of morphologically normal spermatozoa were found in treatment II and III. Morphologically normal spermatozoa in treatment III were significantly (P<0.05) higher than treatment II.

Table 1: Effect of OPN and SNP supplementation on post capacitation morphology of frozen thawed buffalo semen

Groups	Number of experimental animals used for collection of straws	Normal spermatozoa (%±SE)	Abnormal spermatozoa (%±SE)
Control	6	81.75 ^{a} ± 0.16	18.25 ^{a} ± 0.16
Treatment I (OPN-100µg/mL)	6	83.85 ^{a} ± 0.20	16.15 ^{a}± 0.20
Treatment II (SNP-100µM/mL)	6	57.05 ^{b} ± 0.07	42.95 ^{b}± 0.07
Treatment III (OPN- 100µg/mL + SNP 100µM/mL)	6	68.50 ^{c} ± 0.09	31.50 ^{c} ± 0.09

Mean with different superscripts (a, b and c) are significantly different (P<0.05)
Data are presented as mean% ± S.

Discussion
Sperm cell abnormalities were classified based on the location of defects (head, tail and mid piece) or site of origin (primary: testis; secondary: epididymis and tertiary: accessory glands post ejaculation). The significance of specific sperm abnormalities were better understood from the results of mating trials, analysis of non-return rates to artificial insemination and *in vitro* fertilization with semen containing high percentages of sperm with individual classes of abnormalities. Accurate morphological screening of the ejaculates allowed elimination of bulls with low fertility potential, prior to the entrance of bulls to progeny testing program and the preservation of semen, thus contributing to a major saving for Al enterprises [27].
Mammalian spermatozoa membranes are rich in poly unsaturated fatty acids (PUFAs) and are sensitive to oxygen-induced damage mediated by LPO. Thus spermatozoa are sensitive to ROS attack which results in decreased sperm motility, presumably by a rapid loss of intracellular ATP leading to axonemal damage, decreased sperm viability and increased mid-piece sperm morphological defects with deleterious effects on sperm capacitation and acrosomal reaction [18]. Seminal plasma (SP) contains several enzymatic and nonenzymatic antioxidants that confer protection to the sperm against oxidative injuries. However, the removal of SP is a key step for sperm cryopreservation. This step is essential mainly to concentrate the number of spermatozoa. Although this procedure is essential to a successful cryopreservation, removal of SP can increase sperm susceptibility to ROS due to the removal of antioxidant protection system present in seminal plasma [28].
The results of present study showed that the supplementation of SNP caused abnormal morphological changes in spermatozoa. So, less number of normal spermatozoa were found in SNP treatments as compared to control, and other treatments (OPN and combination of SNP with OPN). Previous study also documented that abnormal spermatozoa were increased in presence of high concentration of NO [29]. Several studies demonstrated that a negative correlation existed between NO levels and sperm motility, morphology and DNA fragmentation [30, 31, 32]. Spermatozoa susceptible to ROS attack result in decreased sperm motility by rapid loss of intracellular ATP leading to axonemal damage, decreased sperm viability and increased incidence of midpiece sperm morphological abnormalities [18, 33].
Negative correlation exists between NO concentration and sperm morphology [34, 35]. Spermatozoa treated with SNP and OPN showed significantly lesser number of abnormal spermatozoa than spermatozoa treated with SNP alone. This could be due to down regulation of NO synthesis by OPN [23]. **A**bnormal morphology was higher in young horses, which also showed higher concentrations of OPN [36]. In this study, there was no direct effect of OPN on sperm morphology and no significant change was observed in morphological abnormalities when compared with control.

Conclusions
This study revealed that OPN supplementation partially ameliorates the free radical damage induced by sodium nitroprusside, a NO donor on sperm morphology of frozen thawed buffalo semen.

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