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

Visakh Viswam, K Loganathasamy, VS Gomathy and D Reena

DOI: [https://doi.org/10.22271/j.ento.2020.v8.i6l.7956](https://doi.org/10.22271/j.ento.2020.v8.i6l.7956)

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 strawses from 8 ejaculates in 6 bulls were procured from Central Frozen Sperm Edition and Training Institute, Hessarghatta, Bangalore and stored at Semen Bank, Madras Veterinary College, Chennai. The frozen strawses were thawed and seminal plasma and semen extender were removed from spermatozoa by centrifugation. Spermatozoa were suspended in 1mL capsaication medium (control), addition of 100µg/mL of OPN (treatment I), 100µg/mL of SNP (treatment II) and 100µg/mL of OPN + 100µM/mL of SNP (treatment III). Spernoo with capsaication medium without any treatmment served as control. The contents were incubated at 37°C 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 spermatozoon 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 ONP 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), prostaticand 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, 12]. 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 (O₂⁻), hydrogen peroxide (H₂O₂), peroxyl (ROO⁻) and hydroxyl (OH) radicals [19]. Those derived from nitrogen are called reactive nitrogen species (RNS). RNS include nitric oxide (NO), nitrogen dioxide (NO₂) 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, Bangalore-51. The straws were collected in liquid nitrogen (LN2–196 °C) container, transported and stored in the Semen Bank, Madras Veterinary College, Chennai- 600 007. The frozen straws were thawed at 37°C 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% CO₂ 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].
**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 ± 0.16                | 18.25 ± 0.16                 |
| Treatment I (OPN-100µg/mL) | 6                                                           | 83.85 ± 0.20                | 16.15 ± 0.20                 |
| Treatment II (SNP-100µM/mL) | 6                                                           | 57.05 ± 0.07                | 42.95 ± 0.07                 |
| Treatment III (OPN-100µg/mL + SNP 100µM/mL) | 6                                                           | 68.50 ± 0.09                | 31.50 ± 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 AI 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 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]. Abnormal 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.

**Acknowledgements**

The authors are grateful to Authorities of Tamil Nadu Veterinary and Animal Sciences University for providing necessary funds and facilities to carry out this experiment.

**References**

1. Killian GJ, DA Chapman, LA Rogowski. Fertility associated proteins in Holstein bull seminal plasma. Biol. Reprod 1993;49:1202-1207.
2. Yanagimachi R. Fertility of mammalian spermatozoa: its development and relativity. Zygote 1994;3:371-372.
3. Henault MA, GJ Killian, JF Kavanaugh, LC Griel. Effect of accessory sex gland fluid from bulls of differing fertilities on the ability of cauda epididymal sperm to penetrate zona-free bovine oocytes. Biol. Reprod 1995;52:390-397.
4. Bellin ME, HE Hawkins, JN Oyarzo, RJ Vanderboom, RL Ax. Monoclonal antibody detection of heparin-binding proteins on sperm corresponds to increased fertility of bulls. J. Anim. Sci 1996;74:173-182.
5. Amann RP, RH Hammerstedt, RB Shabanowitz. Exposure of human, boar or bull sperm to a synthetic peptide increases binding to an egg-membrane substrate. J Androl 1999;20:34-41.
6. Fouachecourt S, S Metayer, A Locatelli, F Dacheux, JL Dacheux. Stallion epididymal fluid proteome: qualitative and quantitative characterization; secretion and dynamic changes of major proteins. Biol. Reprod 2000;62:1790-1803.

7. Sprott LR, MD Harris, DW Forrest, J Young, HM Zhang, JN Oyarzo, et al. Artificial insemination outcomes in beef females using bovine sperm with a detectable fertility-associated antigen. J Anim. Sci 2000;78:795-798.

8. McCauley TC, HM Zhang, ME Bellin, RX Ax. Identification of a heparin-binding protein in bovine seminal fluid as tissue inhibitor of metalloproteinases-2. Mol. Reprod. Dev 2001;58:336-341.

9. Moura AA, DA Chapman, GJ Killian. Proteins of accessory sex glands associated with the oocyte-penetrating capacity of cauda epididymal sperm from Holstein bulls of documented fertility. Mol. Reprod. Dev 2006a;74:214-22.

10. Moura AA, DA Chapman, H Koc, GJ Killian. A comprehensive proteomic analysis of cauda epididymal fluid and identification of proteins associated with fertility scores of mature dairy bulls. J Andro 2006b;98(5):71-77.

11. Maxwell WMC, I Parrilla, I Caballero, E Garcia, J Roca, EA Martinez, et al. Retained functional integrity of bull spermatozoa after double freezing and thawing using pure sperm density gradient centrifugation. Reprod. Domest. Anim 2007;42:489-494.

12. Rebollodo AD, LN Sierra, AC Tamayo, AA Loria, SE Denis, RB Oses, et al. Fertility in hair sheep inseminated with freeze spermatozoa diluted with seminal plasma. Rev. Cient. Fac. Cienc. Veter 2007;17:73-76.

13. Garner DL, CA Thomas, CG Gravance, CE Marshall, JM DeJarnette, CH Allen, et al. Seminal plasma addition attenuates the dilution effect in bovine sperm. Theriogenology 2001;56:31-40.

14. Ollero M, N Garcia-Lopez, JA Cebrian-Perez, T Muino-Blanco. Surface changes of ram spermatozoa by adsorption of homologous and heterologous seminal plasma proteins revealed by partition in an aqueous twophase system. Reprod. Fertil. Dev 1997;9:331-390.

15. Martinez-Pastor F, L Anel, C Guerra, M Alvarez, AJ Soler, JJ Garde, et al. Seminal plasma improves cryopreservation of Iberian red deer epididymal sperm. Theriogenology 2006;66:1847-1856.

16. Agarwal A, Saleh R, Bedaiwy MA. Role of reactive oxygen species in the pathophysiology of human reproduction. Fertility and Sterility 2003;79:829-843.

17. Kothari S, A Thompson, A Agarwal, SS Plessis. Free radicals: their beneficial and detrimental effects on sperm function. Indian J. Exp. Biol 2010;48: 425-435.

18. Bansal AK, GS Bilasupri. Impacts of oxidative stress and antioxidants on semen functions. Veterinary Medicine International 2011;4060:1-7.

19. Ford WCL. Regulation of sperm function by reactive oxygen species. Human Reproduction Update 2004;10:387-399. https://doi.org/10.1093/humupd/dmh034

20. Agarwal A, Tamer M, Said MD, Bedaiwy AM, Banerjee J, Alvarez GJ, et al. Oxidative stress in an assisted reproductive techniques setting. Fertility and Sterility 2006;86:503-512.

21. Sikka SC. Relative impact of oxidative stress on male reproductive function. Curr. Med. Chem 2001;8:851-859.

22. Khodaei HR, H Hejazi. Effect of different levels of nitric oxide donor on motility and viability of spermatozoa post thawing in Afshari rams. Research in Agricultural science 2007;2:55-60.

23. Ruffolo Jr RR, ZF Giora, AH Jacqueline, P George, WM Brain. Inflammatory cells and mediators in CNS disease. Taylor and Francis e-library, (Ed.), 2004, pp. 97.

24. Hermawati T, Sri Malyati S, Rimayanti Suprayogi TW. Identification on osteopontin promoter gene polymorphism and post-thawing quality in dairy bull Peranakan Friesian Holstein. In Proceedings of 9th Annual Basic Science International, IOP Publishing 2019.

25. Preedaa MG, Loganathasamy K, Leela V, Pandiyian V. Study on correlation between expression levels of osteopontin gene and in vitro sperm characteristics in bovine semen. Journal of Entomology and Zoology Studies 2020;8:626-630.

26. Enciso M, H Cisaleb, SD Johnstonce, J Sarasaa, JL Fernandezd, G Gosalvez, et al. Major morphological sperm abnormalities in the bull are related to sperm DNA damage. Theriogenology 2011;76:23-32.

27. Padrick P, U Jaakma. Sperm morphology in Estonian dairy bulls, factors affecting it and relation to fertility. Agraarteadu. 2002;13:243-256.

28. Ramires Neto C, Monteiro GA, Soares RF, Pedrazzi C, Dell aqua Jr JA, Papa FO, et al. New seminal plasma removal method for freezing stallion semen. Theriogenology 2013;79:1120-1123.

29. Ambrosini A, G Zolese, S Ambrosi, L Ragni, L Tiano, G Littarru, et al. Oleoylethanolamide protects human sperm cells from oxidation stress: Studies on cases of idiopathic infertility. Biol. Reprod 2006;74:659-665.

30. Balercia G, S Moretti, A Vignini, M Magagnini, F Mantero, M Boscaro, GR Lamonica, et al. Role of nitric oxide concentrations on human sperm motility. J Androl 2004;25:245-249.

31. Huang I, J Jones, O Khorraram. Human seminal plasma nitric oxide: correlation with sperm morphology and testosterone. Med. Sci. Monitor 2006;12:103-106.

32. Amiri I, N Sheikh, R Najafi. Nitric oxide level in seminal plasma and its relation with sperm DNA damages. Iran. Biomed. J 2007;11:259-264.

33. Sikka SC. Oxidative stress and role of antioxidants in normal and abnormal sperm function. Frontiers in Bioscience 1996;1:78-86.

34. Ramya T, MM Misro, D Sinha, D Nandan, S Mithal. Altered level of seminal nitric oxide synthase and enzymatic antioxidants and their association with sperm function in infertile subjects. Fertil. Steril 2011;95:135-140.

35. Vidya G, Garg AT, Rawekar VK, Deshpande DA, Biswas MV Sawane, Akate AN, et al. Effect of oxidative stress on sperm quality in leukocytospermic infertile men. Biomedical Research 2011;22:329-332.

36. Waheed MM, SM El-Bahr, AK Al-haidar. Influence of seminal plasma antioxidants and osteopontin on fertility of the Arabian horse. J Eq. Vet. Sci 2013;33:705-709.