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Visakh Viswam
PG Scholar, Department of Veterinary Physiology, Madras Veterinary College, Chennai, Tamil Nadu, India

K Loganathasamy
Associate Professor and Head, Department of Veterinary Physiology and Biochemistry, Veterinary College and Research Institute, Tirunelveli, Tamil Nadu, India

VS Gomathy
Professor and Head (Retired), Department of Veterinary Physiology, Madras Veterinary College, Chennai, Tamil Nadu, India

D Reena
Assistant Professor, Department of Veterinary Gynaecology and Obstetrics, Madras Veterinary College, Chennai, Tamil Nadu, India

Corresponding Author:
K Loganathasamy
Associate Professor and Head, Department of Veterinary Physiology and Biochemistry, Veterinary College and Research Institute, Tirunelveli, Tamil Nadu, India

Osteopontin ameliorates sodium nitroprusside induced free radical damage on sperm motility of frozen thawed buffalo semen

Visakh Viswam, K Loganathasamy, VS Gomathy and D Reena

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Abstract
The experiment was undertaken to study sodium nitroprusside (SNP) induced free radical damage on sperm motility of frozen thawed buffalo semen and the ameliorative effects of osteopontin (OPN) supplementation on sperm motility. Buffalo frozen semen straws from 8 ejaculates of 6 bulls were procured from Central Frozen Semen Production and Training Institute, Hessarghatta, Banglore and stored at Semen Bank, Madras Veterinary College, Chennai. The semen straws were thawed and seminal plasma and semen extender were removed from spermatozoa by centrifugation. Spermatozoa were suspended in 1mL capacitation medium (control), with the addition of 100µg/mL of OPN (treatment I) or 100µM/mL of SNP (treatment II) or 100µg/mL of OPN + 100µM/mL of SNP (treatment III). Semen with capacitation medium alone without any supplementation served as control. The contents were incubated at 37ºC for 4 h and the post capacitation sperm motility was observed under bright field microscopy. The post capacitation sperm motility was significantly (P<0.05) higher in treatment I (71.80% ± 0.04) as compared to control (61.71% ± 0.03), treatment II (24.82% ± 0.07) and treatment III (46.64% ± 0.05). But, the post capacitation motility in treatment II and III were significantly (P<0.05) lower than control. The post capacitation motility in treatment III was significantly (P<0.05) higher than treatment II. The study indicated that addition of SNP alone in the capacitation medium has detrimental effects on the sperm motility and addition of OPN alone in the capacitation medium exerts beneficial effects on sperm motility. When both OPN and SNP were added in the capacitation medium, OPN partially ameliorated the toxic effects of SNP on the sperm motility of frozen thawed buffalo semen.

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

Introduction
The influence of seminal proteins on male reproduction has drawn attention because many studies proved that their expression is associated with fertility scores in dairy cattle [1], beef cattle [2] and horses [3]. 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 (CLU) and heat shock proteins (HSPs) have been identified in seminal plasma and documented as indicators of fertility [4,5,6,7,8]. OPN is an acidic protein belongs to a family of proteins named SIBLING (small integrin-binding ligand N-linked glycoprotein) [9]. In bulls, OPN in seminal plasma is secreted by ampulla and vesicular gland [10]. High fertility Holstein bulls have greater concentrations of OPN in accessory sex gland fluids than low-fertility bulls [7].In cattle, studies with the use of specific antibodies have shown that OPN is one among the seminal plasma proteins that are associated with fertility. OPN has also been detected at greater concentrations in the seminal plasma than in the sperm cells in buffalo as OPN is produced by ampulla and seminal vesicles, as in case of cattle [11]. Treatment with OPN in the presence of heparin improved sperm *in vitro* sperm capacitation, synchronous pronuclear (PN) formation, blastocyst yield, and embryo quality in buffalo [12]. The importance of OPN in reproduction was also demonstrated in experiments using *in vitro* fertilization [13].
Nitric oxide (NO) has been proved as an inter and intracellular messenger molecule controlling many physiological processes. NO is synthesized from L-arginine by the action of nitric oxide synthase (NOS), an enzyme existing in three isoforms. Two of them, endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS) collectively called as constitutive nitric oxide synthase, are responsible for continuous basal release of NO and both require calcium/calmodulin for activation.

The other one is called as inducible nitric oxide synthase (iNOS), is responsible for prolonged release of NO and does not require calcium/calmodulin for activation. It is expressed in response to inflammatory cytokines and lipopolysaccharides [14, 15, 16]. NO is involved in regulation of mammalian sperm functions such as motility, capacitation and acrosomal reaction [17]. Narrow range of NO concentration enhanced the early events in reproduction. But either the lack of NO or excess of NO had negative consequences [18]. NO produced by sperm NOS participates in capacitation and acrosome reaction of cryopreserved bovine semen [19]. Higher concentration of NO exerts many adverse effects on sperm characteristics like motility, morphology and viability [20].

Motility is one of the most important features of fertile spermatozoa. It was the first and continues to be the most widely used indicator of sperm function. Sperm motility is an important attribute, because it is readily identifiable and reflects several structural and functional competence, as well as essential aspects of spermatozoa metabolism [21]. Hence, the present experiment was undertaken to study influence of both sodium nitroprusside (SNP), a NO donor and OPN, a seminal plasma protein on sperm motility of frozen thawed buffalo semen.

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 of 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 semen 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 post capacitation sperm motility was examined from the above groups as described below.

Table 1: Effects of OPN and SNP supplementation on post capacitation sperm motility of frozen thawed buffalo semen

Groups	Number of experimental animals used for collection of semen straws	Post capacitation Motility (%±SE)
Control	6	61.71 ^{a}± 0.03
Treatment I (OPN-100µg/mL)	6	71.80 ^{b} ± 0.04
Treatment II (SNP-100µM/mL)	6	24.82 ^{c} ± 0.07
Treatment III (OPN- 100µg/mL + SNP 100µM/mL)	6	46.64 ^{d} ± 0.05

Mean with different superscripts (a, b and c) are significantly different (P<0.05)

Data are presented as mean% ± SE.

Evaluation of sperm motility

The sperm motility was assessed by placing a drop of semen from each group on separate clean grease free glass slide and covered with cover slip. Minimum of three fields were scanned under bright field microscopy to assess the per cent progressive motile spermatozoa and graded in terms of per cent ranging from 0-100 in multiples of 10 [22].

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 (Figure-1) shows the effects of OPN and SNP supplementation on post capacitation sperm motility. The post capacitation sperm motility was significantly (P<0.05) higher in treatment I (71.80% ± 0.04) as compared to control (61.71% ± 0.03), treatment II (24.82% ± 0.07) and treatment III (46.64% ± 0.05). The post capacitation motility in treatment II and III was significantly (P<0.05) lower than control. Among the treatment groups, post capacitation sperm motility was significantly (P<0.05) low in treatment II. But, post capacitation motility in treatment III was significantly (P<0.05) higher than treatment II.

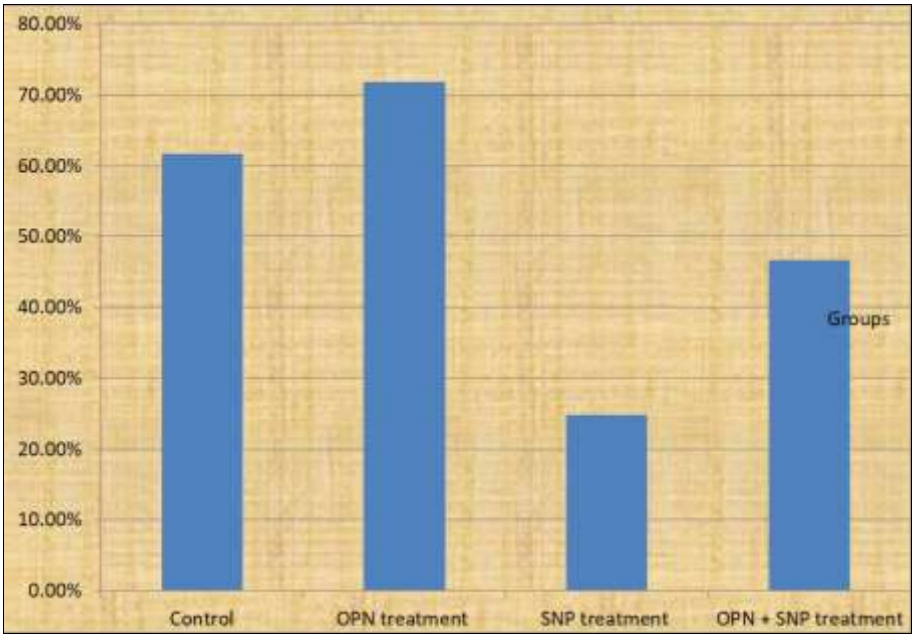

Fig 1: Effects of OPN and SNP supplementation on post capacitation sperm motility of frozen thawed buffalo semen

Discussion

The results of the present study revealed that spermatozoa treated with SNP significantly decreased sperm motility. This study corroborated with several studies which demonstrated that sperm motility was decreased in the presence of different concentrations of SNP [23, 24, 25, 26]. In other studies, NO was shown beneficial for motility of spermatozoa at low concentration but, high concentration was harmful to spermatozoa [27, 28].Variation in sperm motility among the bulls may be due to regulation of nitric oxide (NO) synthesis by OPN because OPN is a general modulator of NO synthesis [29]. Nitric Oxide (NO) is synthesized in epithelial cells of male reproductive tract [25]. NO induces OPN production in macrophage, thereby providing a negative feedback loop to limit NO mediated tissue injury [30]. NO at higher concentration inhibit cellular respiration of spermatozoa by nitrosylation of heme in mitochondrial enzyme, aconitase and glyceraldehydes 3- phosphate dehydrogenase leading to depletion of ATP and consequent loss of motility in the spermatozoa [31]. Spermatozoa obtained from bull semen displaying increased OPN gene expression had high motility than spermatozoa obtained from bull semen with lower gene expression [32]. OPN increases intracellular calcium [33] and thereby increase sperm motility [34]. OPN concentration was significantly high in Arabian horse with higher percentage of motile spermatozoa and they showed a higher fertilization capacity [35]. Spermatozoa treated with SNP and OPN showed significantly higher motility than spermatozoa treated with SNP alone. This could be due to down regulation of NO synthesis by OPN [36].

Conclusions

The study indicated that addition of SNP alone in the capacitation medium has detrimental effects on the sperm motility and addition of OPN alone in the capacitation medium exerts beneficial effects on sperm motility. When both OPN and SNP were added in the capacitation medium, OPN partially ameliorated the toxic effects of SNP on the sperm motility of frozen thawed buffalo semen.

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