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Influence of sodium nitroprusside on sperm functional membrane integrity and acrosome integrity of frozen thawed buffalo semen

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Abstract
The present study was undertaken to assess the effects of exogenous supplementation of sodium nitroprusside (SNP), a nitric oxide (NO) donor on sperm functional membrane integrity and acrosome integrity of frozen thawed buffalo semen. Frozen semen straws from 6 buffalo bulls were procured from Central Frozen Semen Production and Training Institute, Hesseraghatta, Banglore-51. The straws were thawed at 37˚C for 30 seconds and emptied into 15 ml sterile plastic centrifuge tube containing 1 ml of capacitation medium (control), addition of 100 µM SNP to capacitation medium (SNP treatment I) and 100 nM SNP to capacitation medium (SNP treatment II) were incubated at 37˚C for 1 hour. After 1 hour incubation, sperm functional membrane integrity was studied by hypo-osmotic swelling test (HOST). In control, SNP treatment I and II, 57.42% ± 0.62, 28.08% ± 1.02 and 39.75% ± 1.20 spermatozoa had intact functional membrane. Functional membrane integrity of spermatozoa was significantly (P<0.01) higher in control than SNP treatment I and II. Spermatozoa with intact functional membrane were significantly (P<0.01)more in SNP treatment II than SNP treatment I. Sperm acrosome integrity was assessed by Giemsa stain method. Acrosome intact spermatozoa were significantly (P<0.01) more in control (37.42% ± 0.70) as compared to SNP treatment I (12.58% ± 0.68) and II (27.33% ± 0.64). Spermatozoa with intact acrosome were significantly (P<0.01) high in SNP treatment II than SNP treatment I. From this study, it is concluded that addition of SNP, a NO donor has detrimental effects on sperm functional membrane integrity and acrosome integrity of frozen thawed buffalo semen on concentration dependant manner.

Keywords: Sperm functional membrane integrity, acrosome integrity

Introduction
Oxidative stress (OS) is an imbalance between pro-oxidative and anti-oxidative molecules in a biological system. It arises as a consequence of excessive production of free radicals and impaired antioxidant defense mechanism [1,2,3].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 [4].Those free radicals derived from nitrogen are called reactive nitrogen species (RNS). RNS include nitric oxide (NO^-), nitrogen dioxide (NO_2) and peroxynitrite anion($ONOO^-$)[5,6].RNS are often considered to be subclass of ROS [7].Free radical may have beneficial or detrimental effects on sperm functions depending on their nature and concentration [8,9].

Recent evidence on the NO has proved its importance as an intercellular and intracellular messenger controlling many physiological processes. It is also a mediator of cytokines and growth factors in various cell types. NO is synthesized form 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 (cNOS), 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 [10].

Male fertility markers have been scrutinized in order to comprehend the molecular events that can lead to subfertility and permit an accurate diagnosis and design of therapeutic protocols. Among these markers, the study of oxidative stress (OS) in semen has emerged as promising field.

Recently NO has emerged as a potent regulator which controls the sperm functions. Low concentration of NO increased the motility and viability of spermatozoa. However, high concentration of NO decreased the sperm motility and viability in ram [11]. But studies on effects of NO on *in vitro* sperm characteristics are scarce. Hence the present study was undertaken to study the effect of sodium nitroprusside (SNP), a nitric oxide (NO) donor on sperm functional membrane integrity and acrosome integrity of frozen thawed buffalo semen.

Materials and Methods

Materials

Laboratory Chemicals

Tris buffer, phosphate buffer saline (PBS), thiobarbituric acid and trichlor acetic acid (TBA-TCA) solution, sodium pyruvate, sodium chloride, sodium bicarbonate, sodium phosphate, sodium lactate, potassium chloride, calcium chloride, magnesium chloride, heparin, fatty acid free bovine serum albumin (BSA), penicillin-G, streptomycin and phenol red were procured from Sigma chemicals Co., USA.

Plastic ware and Glassware

All the plastic ware used for research *viz*., test tube, centrifuge tube, micro centrifuge tube, micro tips etc. were purchased from Falcon, New Jersey, USA. All the glassware used for research *viz*., laboratory bottles, microscope cover slip 18 mm x 18mm, microscope slide with ground edges 25.4 x 76.2 mm etc. were purchased from Borosil, India.

Methods

Collection of Semen straws

Frozen buffalo semen straws from 6 bulls were procured from Central Frozen Semen Production and Training Institute, Hesseraghatta, Banglore-51.The straws were transported in liquid nitrogen (LN_2) container (-196 º C) and kept in semen bank of Madras Veterinary College, Chennai – 7.

Sperm preparation

The frozen semen straws were thawed at 37˚C for 30 seconds and emptied into 15 ml sterile centrifuge tube containing 1 ml of capacitation medium alone (control),capacitation medium with SNP @100 µM (SNP treatment-I) and capacitation medium with SNP @100 nM (SNP treatment-II).The contents were incubated at 37°C for 1 hour. After 1 hour incubation, the following parameters were assessed from the above groups.

Evaluation of sperm functional membrane integrity

100µl semen from each group was taken in a separate Eppendorff tube and 500µl distilled water was added and mixed properly. This solution was kept at 37˚C for 1 hour. After 1 hour incubation, 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 with intact functional membrane showed coiled tail and those without intact functional membrane showed straight tail. A minimum of 200 spermatozoa were observed [12].

Evaluation of sperm Acrosomal integrity

One drop of semen from each group was taken on a separate clean grease free glass slide and smear was prepared. The slide was air dried and the slide was kept in the working solution of Giemsa stain for 6 hours. Stain was washed with tap water and air dried. Spermatozoa were observed under oil objective of the microscope. Acrosomal intact spermatozoa showed acrosomal cap and acrosomal nonintact spermatozoa lost acrosomal cap. A minimum of 200 spermatozoa were observed [13]

Statistical analysis

Statistical analysis was carried out by Completely Randomized Design (CRD) described [14].

Results

Effect of no on sperm Functional Membrane Integrity

The sperm functional membrane integrity was studied by hypo-osmotic swelling test (HOST). It is evident from the table 1 that in control, SNP treatment I and II, 57.42% ± 0.62, 28.08% ± 1.02 and 39.75% ± 1.20 spermatozoa had intact functional membrane. Functional membrane integrity of spermatozoa was significantly (P<0.01) higher in control than SNP treatment I and II. Spermatozoa with intact functional membrane were significantly (P<0.01) more in SNP treatment II than SNP treatment I.

Effect of no on sperm Acrosomal integrity

The sperm acrosomal integrity was assessed by Giemsa stain method. Table 2 shows that acrosomal intact spermatozoa were significantly (P<0.01) more in control (37.42% ± 0.70) as compared to SNP treatment I (12.58% ± 0.68) and II (27.33% ± 0.64). Spermatozoa with intact acrosome were significantly (P<0.01) high in SNP treatment II than SNP treatment I.

Table 1: Effect of SNP Supplementation on Post Capacitation Functional Membrane Integrity of Frozen Thawed Buffalo Semen

3	Number of animals used for collection of semen	Functional membrane intact spermatozoa (% ± SE)	Functional membrane nonimpact spermatozoa (% ± SE)
Control	6	57.42^{a} ± 0.62	42.58^{a} ± 0.62
SNP treatment I	6	28.08^{b} ± 1.02	71.91^{b} ± 1.02
SNP treatment II	6	39.75^{c} ± 1.20	60.25^{c} ± 1.20

Means with different superscripts (a, b and c) are significantly different (P<0.01) Data are presented as mean % ± S. E.

Table 2: Effect of SNP Supplementation on Post Capacitation Acrosomal Integrity of Frozen Thawed Buffalo Semen

Groups	Number of animals used for collection of semen	Acrosome intact spermatozoa (% ± SE)	Acrosome nonimpact spermatozoa (% ± SE)
Control	6	37.42^{a} ± 0.70	62.58^{a} ± 0.70
SNP treatment I	6	12.58^{b} ± 0.68	87.42^{b} ± 0.68
SNP treatment II	6	27.33^{c} ± 0.64	72.67^{c} ± 0.64

Means with different superscripts (a, b and c) are significantly different (P<0.01) Data are presented as mean % ± S. E.

Discussion

Effect of no on sperm functional membrane integrity

The sperm plasma membrane is largely composed of PUFAs which are susceptible to oxidative damage due to the existence of double bond [15]. The sperm membrane contains almost 50 per cent docosahexaenoic acid, which contains six unsaturated double bonds in every molecule. As the LPO cascade proceeds in sperm, almost 60% of the fatty acid is lost from the membrane. LPO affects membrane structure and functions such as fluidity, ion gradients, receptor transduction, transport processes and membrane enzymes [16]. Peroxides, products of LPO constitute a potential hazard to the structural and functional integrity of spermatozoa [17]. As a result, functions that are necessary for normal fertilization are impaired [18].

Excessive NO contributes to the formation of peroxynitrite, a highly toxic anion of peroxidation. Peroxynitrite is not a free radical because the unpaired electrons of NO and superoxide combine to form a new nitrogen-oxygen bond in peroxynitrite, but it is a strong one or two-electron oxidant and nitrating agent. Even at physiological pH, the relative stability of peroxynitrite allows it to diffuse for a considerable distance on a cellular scale and even to cross cell membranes. Peroxynitrite can react rapidly with lipids, protein and DNA and exerts its cytotoxic effect [19].

Peroxyl radicals are toxicologically relevant species since they are involved not only in the disruption of cell membrane integrity, but also in the oxidation of membrane lipids, proteins, inactivation of receptors and membrane-bound enzymes [20]. In this study, spermatozoa exposed to SNP were more prone to LPO. So, more spermatozoa lost their functional membrane integrity depending on the concentration.

Effect of no on Sperm Acrosomal Integrity

The results of the present study differ with previous study [21]. They reported that there was no detectable decrease in acrosomal integrity due to generation of ROS through the use of the xanthine (X) – xanthine oxidase (XO) system. In this study, the spermatozoa showed lower acrosomal integrity when exposed to SNP in this study. Differences in generation of types of ROS especially, NO may be paramount reason for spermatozoa with less acrosome integrity in SNP treatments (I and II). Differences in experimental methodology may account for different observations regarding the effect of ROS generated. Likewise, there may be species differences in susceptibility of spermatozoa to OS or perhaps may be species differences in the antioxidant capacity associated with spermatozoa.

Conclusion

This study indicated that addition of SNP, a NO donor has detrimental effects on sperm functional membrane integrity and acrosome integrity of frozen thawed buffalo semen on concentration Dependent manner.

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