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RESEARCH ARTICLE

Influence of sodium nitroprusside on sperm motility, viability and morphology of frozen thawed buffalo semen

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Abstract : The present investigation has been undertaken to study the effects of exogenous supplementation of sodium nitroprusside (SNP), a nitric oxide (NO) donor on *in vitro* sperm characteristics of buffalo semen. Buffalo straws from 6 bulls were procured from Central Frozen Semen Production and Training Institute, Hesseraghatta, Banglore-51. The frozen 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/ml of SNP (SNP treatment I) and 100 nM/ml of SNP (SNP treatment II) and were incubated at 37°C for 1 hour. After 1 hour incubation, the progressive motility was studied under bright field microscopy. Sperm motility was significantly (P<0.01) lowered in SNP treatment I (11.67% ± 1.67) and II (23.33% ± 2.11) as compared to control (43.33% ± 2.11). Between treatments, sperm motility was significantly (P<0.01) high in SNP treatment II than SNP treatment I. The sperm viability was assessed by the supravital Eosin and Nigrosin stain method. Significantly (P<0.01) higher percentage of spermatozoa were alive in control (56.67% ± 0.88) in comparison with SNP treatment I (25.08% ± 1.19) and II (37.83% ± 1.27). But,more spermatozoa were significantly (P<0.01) alive in SNP treatment II than SNP treatment I. The sperm morphology was determined by Rose Bengal stain technique. Morphologically normal spermatozoa were significantly (P<0.01) more in control (83.00% ± 0.62) when compared to SNP treatment I (53.00% ± 0.59) and II (68.42% ± 0.87). In similar way, significantly (P<0.01) higher proportions of spermatozoa were morphologically normal 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 the sperm motility, viability and morphology of frozen thawed buffalo semen on concentration dependant manner.

Key words : Sperm motility, viability, Morphology, Sodium nitroprusside, Buffalo semen

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INTRODUCTION

Oxidative stress (OS) is emerging as 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 (Agarwal *et al.,* 2003; Kothari *et al*., 2010 and Bansal and Bilaspuri, 2011).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 (Ford, 2004).Those derived from nitrogen are called reactive nitrogen species (RNS). RNS include nitric oxide (NO^-), nitrogen dioxide (NO_2) and peroxynitrite anion ($ONOO^-$) (Armstrong *et al*., 1999 and Agarwal *et al*., 2006). RNS are often considered to be subclass of ROS (Sikka, 2001). Free radical may have beneficial or detrimental effects on sperm functions depending on their nature and concentration (Baker *et al.,* 2003). Functional sperm parameters are affected by ROS including hydrogen peroxide, superoxide anion and hydroxyl radicals (Aitken, 1995).

Recent information 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 (Moncada *et al*., 1991).

NO has been found in several cell types. It regulates the vascular tone, neurotransmission and host defense mechanism. NO brings its action by binding with ion containing enzyme and regulates in both positive and negative pathways *i.e.* cGMP dependent and cGMP independent pathways (Nathan, 1992).

In vitro capacitation of spermatozoa is one of the major steps of *in vitro* embryo production (IVEP). *In vitro* capacitation can be improved by supplementation of additives like heparin, albumin and various anti-oxidants. 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 (Khodaei and Hejazi, 2007). But studies on effects of NO on *in vitro* capacitation of buffalo sperm are scarce. Hence the present study was undertaken to study the effect of sodium nitroprusside (SNP), a nitric oxide (NO) donor on sperm motility, viability and morphology of frozen thawed buffalo semen.

RESEARCH METHODOLOGY

Laboratory chemicals :

Eosin stain, Nigrosin stain, Rose Bengal stain, Tris buffer, Phosphate buffer saline (PBS), Thiobarbituric acid and Trichlor acetic acid (TBA-TCA) solution, JC-1 stain (5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolylcarbocyanine iodide), Carboxy fluoresin diacetate (CFDA), Propidium iodide (PI), 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.

Plasticware and glassware :

All the plasticware 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.

Collection of semen straws :

Buffalo 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 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/ml(SNP treatment-I) and capacitation medium with SNP @100 nM/ml(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 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 a 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 (Bansal and Bilaspuri, 2008).

Evaluation of live and dead sperm :

The sperm viability was determined by the supravital Eosin and Nigrosin stain technique. A drop of semen from each group was placed on a separate clean grease free glass slide. One drop of 5 per cent Eosin and 2 drops of 10 per cent Nigrosin near the semen drop on either side were placed. By using a blunt glass rod Eosin was mixed with semen and then Nigrosin was mixed with semen gently. A thin homogenous smear was prepared from semen-stain mixture and dried in air and examined under oil objective of the microscope. Spermatozoa which were unstained (white) classified as live and stained with pink colour classified as dead. At least 200 spermatozoa were counted in each sample (Bjorndahl *et al*., 2003).

$$\textbf{Live percentage} = \frac{\textbf{Live sperm counted}}{\textbf{Total sperm counted}} \textbf{ x 100}$$

Evaluation of normal and abnormal sperm :

Sperm morphology was assessed by Rose Bengal stain. 250 µl semen from each group was emptied into a separate Eppendorff tube containing 500 µl of Tris buffer and then 3 drops of Rose Bengal stain was added. The contents were centrifuged at 2000-3000 rpm for 3 minute. Then 1 ml Tris buffer was added. Again the contents were centrifuged at 2000-3000 rpm for 3 minute. Supernatant was removed. Then 100 µl Tris buffer was added. One drop of the well mixed sample was placed on a glass slide and covered with cover slip. Spermatozoa were observed under 40x. Spermatozoa showing head, midpiece and tail abnormalities were included in total abnormalities. A minimum of 200 spermatozoa were observed (Enciso *et al*., 2011).

Statistical analysis :

Statistical analysis was carried out by Completely Randomized Design (CRD) described by Snedecor and Cochran (1994).

RESULTS AND DISCUSSION

The results obtained from the present investigation as well as relevant discussion have been summarized under following heads :

Effect of NO ON sperm motility :

After 1 hour incubation, the progressive motility was studied under bright field microscopy. Table 1 shows that effect of SNP supplementation to sperm TALP on sperm motility. Sperm motility was significantly ($P<0.01$) lowered in SNP treatment I (11.67% ± 1.67) and II (23.33% ± 2.11) as compared to control (43.33% ± 2.11). Between treatments, sperm motility was significantly ($P<0.01$) high in SNP treatment II than SNP treatment I.

Effect of NO ON sperm viability :

The sperm viability was assessed by the supravital Eosin and Nigrosin stain method. Table 2 depicts that

significantly (P<0.01) higher percentage of spermatozoa were alive in control (56.67% ± 0.88) in comparision with SNP treatment I (25.08% ± 1.19) and II (37.83% ± 1.27). But in SNP treatment II, more spermatozoa were significantly (P<0.01) alive than SNP treatment I.

Effect of NO ON sperm morphology :

The sperm morphology was determined by Rose Bengal stain technique. Table 3 indicates that morphologically normal spermatozoa were significantly (P<0.01) more in control (83.00% ± 0.62) when compared to SNP treatment I (53.00% ± 0.59) and II (68.42% ± 0.87). In similar way, significantly (P<0.01) higher proportions of spermatozoa were morphologically normal in SNP treatment II than SNP treatment I.

Effect of NO ON sperm motility :

The results of the present study revealed that spermatozoa treated with SNP significantly decreased sperm motility in dose/concentration dependent manner in comparison with control.Our study corroborated with several studies who demonstrated that sperm motility was decreased in presence of different concentrations of SNP (Tomlinson *et al*., 1995; Herrero *et al*., 1994; Rosselli *et al*., 1995 and Weinberg *et al*., 1995).In contrast, other reports showed that low concentration of NO enhanced the sperm motility in mouse (Hellstrom *et al*., 1994). The results were further supported by findings of Rosselli *et al*. (1998) who reported that exogenous NO was beneficial at low concentration for the motility of spermatozoa but, high concentration was harmful to spermatozoa. This bimodal motility response to various concentrations of NO releasing compounds could be due to dual nature of NO as both a transduction molecule at low concentration and a cytotoxic effector at high concentrations in systems (Herrero and Gagnon, 2001).

Reduction in sperm motility may also be contributed by the over production of free radical and consequent exposure to oxidative conditions as reported by Balercia *et al*. (2004). They suggested that NO might inhibit cellular respiration by nitrosylation of heme in mitochondrial enzymes, aconitase and glyceraldehyde-3- phosphate dehydrogenase, leading to a depletion of ATP and consequent loss of motility in the spermatozoa. Hyslop *et al*. (1998) reported that free radicals cause perturbations in important biochemical functions, including increased formation of oxidized intracellular sulfhydryls, rapid decrease in ATP levels and a consequent depression of glycolytic

Table 1 : Effect of snp supplementation on post capacitation motility of frozen thawed buffalo semen

Groups	Number of experimental animals	Post capacitation motility (% ± SE)
Control	6	$43.33^{a} \pm 2.11$
SNP treatment I	6	$11.67^{b} \pm 1.67$
SNP treatment II	6	$23.33^{c} \pm 2.11$

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 viability of frozen thawed buffalo semen

Groups	Number of experimental animals	Live spermatozoa (% ± SE)	Dead spermatozoa (% ± SE)
Control	6	$56.67^{a} \pm 0.88$	$43.33^{a} \pm 0.88$
SNP treatment I	6	$25.08^{b} \pm 1.19$	$74.92^{b} \pm 1.19$
SNP treatment II	6	$37.83^{c} \pm 1.27$	$62.17^{c} \pm 1.27$

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

Table 3 : Effect of snp supplementation on post capacitation morphology of frozen thawed buffalo semen

Groups	Number of experimental animals	Normal spermatozoa (% ± SE)	Abnormal spermatozoa (% ± SE)
Control	6	$83.00^{a} \pm 0.62$	$17.00^{a} \pm 0.62$
SNP treatment I	6	$53.00^{b} \pm 0.59$	$47.00^{b} \pm 0.59$
SNP treatment II	6	$68.42^{c} \pm 0.87$	$31.58^{c} \pm 0.87$

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

flux.Accordingly, de Lamirande and Gagnon (1992 a and b) suggested that inhibition of sperm motility after incubation with ROS was caused by depletion of sperm ATP. These authors proposed that sperm immobilization was due to the decreased phosphorylation of axonemal proteins required for sperm movement. Further investigations indicated that ROS inhibited one or more enzymes of oxidative phosphorylation, glycolysis or both, thus, limiting ATP generation by sperm cell.

Mammalian spermatozoal membranes are rich in polyunsaturated 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 (Sikka, 1996; Bansal and Bilaspuri, 2007 and Bansal and Bilaspuri, 2011). Initially it was believed that NO reaches its intracellular targets by freely diffusing through the cell membranes. However, because of its ultra-short half-life (<30 s) and high reactivity with superoxide, heme and non-heme-iron, the concept of free diffusion of NO was hard to accept. Because NO reacts with thiol (-SH) groups of proteins, it can form stable, biologically active, *S*-nitrosyl compounds (Stamler *et al.*, 1992). Indeed, NO has been shown to circulate as a *S*-nitroso adduct of albumin and possess biological activities identical to those of NO *in vitro* and *in vivo* (Keaney *et al.*, 1993). Since iron-nitrosyl compounds are associated with thiol-containing ligands, formation of dinitrosyl iron cysteine has been suggested as a second pathway for NO transport (Mulsch *et al.*, 1993).

NO is now known to mediate multiple biological effects, although initially the effects of NO were thought to be solely mediated via activation of soluble guanylate cyclase and cyclic guanosine 3',5'-monophosphate (cGMP) (Murad, 1994). However, recent studies suggest that NO can also induce its biological effects via non-cGMP-dependent pathways. Since NO is paramagnetic, it is capable of forming high-affinity-nitroso complexes with a variety of metal complexes (Lancaster *et al.*, 1992). NO binds to the heme-containing proteins, for example oxyhaemoglobin, and to iron-sulphur-containing proteins of the tricarboxylic acid cycle enzyme *cis*-aconitase. NO can also bind to the thiol (-SH) groups of glyceraldehyde-3-phosphate dehydrogenase (GADPH) and via this mechanism NO is thought to decrease glycolytic activity associated with myocardial stunning, reperfusion injury and neurotoxicity and to inhibit mitochondrial respiration (Moncada *et al.*, 1991 and Kelly *et al.*, 1996). Other relevant mechanisms may include *S*-nitrosylation of thiols, formation of nitrotyrosine, NO binding to iron-sulphur clusters and NO binding to heme-containing proteins of the respiratory chain (McDonald and Moss, 1993 and Mohr *et al.*, 1996).

Effect of NO ON sperm viability :

The results of the present study showed that the supplementation of SNP with increasing concentrations decreased the sperm viability. So, less number of spermatozoa were found alive in SNP treatments as compared to control. NO can act as a free radical scavenger and inactivate superoxide, thereby preventing cell toxicity at low concentration (Cook and Tsao, 1993). However, under high concentration, reaction of NO with superoxide can also result in the generation of peroxynitrite, a potent oxidant (Beckman *et al.*, 1990 and Beckman and Crow, 1993). Peroxynitrite decomposes to form the reactive hydroxyl radical. Moreover, peroxynitrite and its metabolite are capable of inducing cytotoxicity by inducing LPO, nitrosation of several tyrosine molecules that regulate enzyme function and signal transduction and sodium channel inactivation. Together, these findings suggest that the actions of NO in a cell depend on its concentration, the cellular redox state, and the abundance of metals, protein thiols and low-molecular weight thiols (glutathione), as well as other nucleophil targets (Davies *et al.*, 1995 and Kelly *et al.*, 1996).

Effect of NO ON sperm morphology :

The results of the present study showed that the supplementation of SNP with increasing concentrations causes abnormal morphological changes in spermatozoa. So, less number of normal spermatozoa were found in SNP treatments as compared to control.Several studies endorsed our findings that a negative correlation existed between NO levels and sperm motility, morphology and DNA fragmentation (Balercia *et al*., 2004; Amiri *et al*., 2006; Huang *et al*., 2006 and Amiri *et al*., 2006).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 midpiece sperm morphological

abnormalities (Sikka, 1996; Bansal and Bilaspuri, 2007 and Bansal and Bilaspuri, 2011).Vidya *et al*. (2011) suggested that possible role of NO in male infertility. They found a negative correlation between NO concentrations and sperm morphology.Our study is also supported by Ambrosini *et al*. (2006) who demonstrated that abnormal spermatozoa were increased in presence of high concentration of NO. Ramya *et al*. (2011) found a similarity with our findings that high level of NO result in abnormalities in the spermatozoa.

This present study indicates that addition of SNP, a NO donor has detrimental effects on the sperm motility, viability and morphology of frozen thawed buffalo semen on concentration dependant manner.

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