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Effect of sodium nitroprusside on sperm lipid peroxidation status of frozen thawed buffalo semen

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Abstract
The experiment was conducted to assess the effect of exogenous supplementation of sodium nitroprusside (SNP), a nitric oxide (NO) donor on lipid peroxidation (LPO) status 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) and incubated at 37 ˚C for 1 hour. After incubation, sperm LPO status was measured by production of malondialdehyde (MDA) level. The MDA levels were significantly (P<0.01) low in control (1.48 µmol/ml ± 0.12) when compared to SNP treatment I (3.79 µmol/ml ± 0.07) and II (2.76 µmol/ml ± 0.06). Between treatments, MDA level was low in SNP treatment II than SNP treatment I. This study indicates that addition of SNP in the capacitation medium increases sperm LPO in frozen thawed buffalo semen in concentration dependent manner.

Keywords: Sperm lipid peroxidation, malondialdehyde, sodium nitroprusside, buffalo semen

Introduction
Free radicals are powerful initiators of lipid peroxidation (LPO) at higher concentration and impair human sperm function through damages in membrane fluidity and integrity [1].Free radicals may have beneficial or detrimental effects on sperm functions depending on their nature and concentration [2]. 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 [3]. Those free radicals derived from nitrogen are called reactive nitrogen species (RNS). RNS include nitric oxide (NO^-), nitrogen dioxide (NO_2) and peroxynitrite anion ($ONOO^-$) [4, 5]. RNS are often considered to be subclass of ROS [6].
NO is an important intercellular and intracellular messenger involved in 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 [7].
NO may normally be useful for maintaining sperm motility but can be toxic in excess [8]. Other RNS such as nitrogen dioxide radical and peroxynitrite anion are considered to be damaging to sperm cells. The primary mechanism of NO induced sperm damage is likely to be the inhibition of mitochondrial respiration and DNA synthesis [9]. NO induced toxicity is also mediated indirectly through its interaction with superoxide anions and formation of peroxynitrite anion, which decomposes to form hydroxyl and nitrogen dioxide radicals, both of which are cytotoxic agents [10]. Malondialdehyde (MDA) is an end product of LPO. The acyl chains of decosahexaenoic acid (DHA) bound to phospholipids of sperm membrane are particularly susceptible to ROS/RNS attack leading to the formation of MDA [11]. Hence, this study was undertaken to assess the effect of sodium nitroprusside (SNP), a nitric oxide (NO) donor on sperm LPO status in 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.

Plasticware and glassware

All the plasticware used in this experiment viz., test tube, centrifuge tube, micro centrifuge tube, micro tips etc. were purchased from Falcon, New Jersey, USA and All the glassware 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 ^oC) 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 incubation, LPO status was measured as detailed below.

Determination of sperm LPO status

LPO level of spermatozoa was estimated in semen samples by measuring MDA production using TBA with slight modifications in sperm concentration and incubation time. The semen was thawed and washed twice in Tris buffer by centrifugation at 500 g for 5 min. Then the sperm pellet was re-suspended in 1 ml of PBS (pH 7.2) or a variable volume of PBS to obtain a sperm concentration of 30×10^6/ml. Lipid peroxide level was measured in spermatozoa after the addition of 2 ml of TBA-TCA reagent (15% w/v TCA and 0.375% w/v TBA in 0.25N HCl) to 1 ml of spermatozoa suspension. The mixture was kept in a boiling water bath for 45 min. After cooling, the suspension was centrifuged at 500 g for 15 min. The supernatant was separated and the absorbance measured at 535 nm under UV spectrophotometer (Cecil CE 2021, 2000 series). The MDA concentration was determined by the specific absorbance coefficient (1.56×10^5/molcm^{-3}) [12].

$$\text{MDA (µmol/ml)} = \frac{\text{OD} \times 10^6 \times \text{total volume (3ml)}}{1.56 \times 10^5 \times \text{test volume (1ml)}} = \frac{\text{OD} \times 30}{1.56}$$

Statistical analysis

Statistical analysis was carried out by Completely Randomized Design (CRD) described by Snedecor and Cochran [13].

Results

Sperm LPO status was assessed by estimation of malondialdehyde (MDA) production. The MDA level in frozen thawed capacitated buffalo semen samples of different treatments are presented in Table 1. The MDA levels were significantly (P<0.01) low in control (1.48 µmol/ml ± 0.12) when compared to SNP treatment I (3.79 µmol/ml ± 0.07) and II (2.76 µmol/ml ± 0.06). Between treatments, MDA level was low in SNP treatment II than SNP treatment I.

Table 1: Effect of snp supplementation on post capacitation sperm lipid peroxidation status of frozen thawed buffalo semen

Groups	Number of experimental animals used for collection of semen straws	Malondialdehyde (µmol/ml ± SE)
Control	6	1.48^a ± 0.12
SNP treatment I	6	3.79^b ± 0.07
SNP treatment II	6	2.76^c ± 0.06

Means with different superscripts (a, b and c) are significantly different (P<0.01)

Data are presented as mean µmol/ml ± S. E.

Discussion

Sperm plasma membrane is mainly composed of PUFAs which are susceptible to oxidative damage due to the existence of double bond [14]. The sperm membrane contains almost 50 per cent decosahexaenoic 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 [15]. Peroxides, products of LPO constitute a potential hazard to the structural and functional integrity of spermatozoa [16]. As a result, functions that are necessary for normal fertilization are impaired [17].

Excessive NO contributes to the formation of peroxynitrite, a highly toxic anion of peroxidation. 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 [18].

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 [19].

There are many pathological effects of LPO on sperm function. Overall, LPO damages DNA and proteins through oxidation from lipid peroxyl or alkoxyl radicals. DNA damage by LPO can occur via base modifications, strand breaks or cross-linking [20]. In addition, LPO can result in a loss in membrane fluidity and as a result decreased sperm motility and sperm-oocyte fusion [21].

The extent of this damage depends on many factors including duration of exposure, type of ROS and concentration as well as external factors like temperature, oxygen tension and antioxidant levels [22].

Amongst ROS and RNS, peroxynitrite resulting from the diffusion controlled reaction of NO and superoxide and peroxyl radicals resulting from LPO radical chain reactions are important cellular components of oxidative stress. These are the possible reasons for increased MDA level in SNP treatments (I and II) than control in this study.

Conclusion

This study indicates that addition of SNP in the capacitation medium increases lipid peroxidation of sperm in frozen thawed buffalo semen in concentration dependent manner.

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