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Influence of sodium nitroprusside on sperm mitochondrial membrane potential of frozen thawed buffalo semen

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Abstract
The present investigation was conducted to study the effect of exogenous addition of sodium nitroprusside (SNP), a nitric oxide (NO) donor on sperm mitochondrial membrane potential (MMP) 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 MMP was determined by JC-1(5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolylcarbocyanine iodide) stain technique. Significantly (P<0.01) higher percentage of spermatozoa showed MMP in control (34.33% ± 1.31) in comparison with SNP treatment I (12.50% ± 0.80) and II (22.17% ± 0.61). Significantly (P<0.01) high numbers of spermatozoa showed MMP in SNP treatment II than SNP treatment I. This study indicates that addition of SNP in the capacitation medium decreases sperm MMP in frozen thawed buffalo semen in concentration dependant manner.

Keywords: Mitochondrial membrane potential, spermatozoa, buffalo semen

Introduction
Free radicals may have beneficial or detrimental effects on sperm functions depending on their nature and concentration [1]. 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 [2]. Those free radicals derived from nitrogen are called reactive nitrogen species (RNS). RNS include nitric oxide (NO^-), nitrogen dioxide (NO_2) and peroxynitrite anion ($ONOO^-$) [3, 4]. RNS are often considered to be subclass of ROS [5].
RNS such as NO, NO_2 and peroxynitrite anion are considered to be damaging molecules to sperm cells. The primary mechanism of NO induced sperm damage is likely to be the inhibition of MMP and DNA synthesis [6]. NO induced toxicity is also mediated indirectly through its interaction with superoxide and formation of peroxynitrite which decreases MMP [7]. Simultaneous formation of NO and superoxide produces peroxynitrite, a very strong oxidant and nitrating agent. This is capable of inhibiting important mitochondrial enzymes and affecting MMP of spermatozoa [8]. NO affects the mitochondrial function by interaction with low molecular weight thiols such as glutathione and protein thiols [9].
NO regulates the mitochondrial function by binding to cytochrome c oxidase, the terminal enzyme in the electron transport chain. It competes with oxygen, inhibiting the activity of the enzyme and thus negatively regulating mitochondrial oxidative phosphorylation particularly at the low oxygen concentrations in the spermatozoa [10].
Hence, the experiment was undertaken to assess the effect of sodium nitroprusside (SNP), a nitric oxide (NO) donor on sperm MMP in frozen thawed buffalo semen.

Materials and methods
Materials
Laboratory chemicals: Sodium nitroprusside, 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: Plasticware used in this experiment viz., test tube, centrifuge tube, micro centrifuge tube, micro tips etc. were purchased from Falcon, New Jersey, USA and 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, sperm MMP was measured as detailed below.

3.2.8 Evaluation of sperm MMP: Mitochondrial membrane potential was assessed by using JC-1 (5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolylcarbocyanine iodide) stain technique. 1.53 mM of JC-1 in dimethyl sulfoxide (DMSO), 8.69 mM of CFDA in DMSO and 0.4 mM of PI in PBS were prepared and stored at -20 °C in dark. 2 µl of JC-1 and 10 µl of CFDA solutions were added to 100 µl of semen sample. The semen sample from each group was separately incubated at room temperature for 30 min in dark. The sperm nuclei were counterstained by adding 10 µl of PI stock solution and incubated in dark for 10 min. Then, sperm cells were washed in PBS by centrifugation at 560 g for 5 min. Sperm cells suspended in PBS were placed on a glass slide and covered with cover slip and observed under fluorescent microscope. JC-1 existed as a monomer with excitation and emission peaks in the green wave lengths (510-520 nm). However, it also exhibited a second peak in the red-orange range (590 nm). Spermatozoa with high MMP exhibited red-orange fluorescence and those with low to medium MMP exhibited green fluorescence. Spermatozoa without MMP did not exhibit fluorescence. A minimum of 200 spermatozoa were observed [11].

Statistical analysis: Statistical analysis was carried out by Completely Randomized Design (CRD) described by Snedecor and Cochran [12].

Results

Table 1 depicts that significantly (P<0.01) higher percentage of spermatozoa showed MMP in control (34.33% ± 1.31) in comparison with SNP treatment I (12.50% ± 0.80) and II (22.17% ± 0.61). Significantly (P<0.01) high numbers of spermatozoa showed MMP in SNP treatment II than SNP treatment I.

Table 1: Effect of sodium nitroprusside supplementation on post capacitation sperm mitochondrial membrane potential of frozen thawed buffalo semen

Groups	Number of experimental animals used for collection of semen straws	Spermatozoa with MMP (% ± SE)	Spermatozoa without MMP (% ± SE)
Control	6	34.33^{a} ± 1.31	65.67^{a} ± 1.31
SNP treatment I	6	12.50^{b} ± 0.80	87.50^{b} ± 0.80
SNP treatment II	6	22.17^{c} ± 0.61	77.83^{c} ± 0.61

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

Discussion

ROS may also adversely affect sperm motility via alterations in mitochondrial function. MMP has been used as a measure of mitochondrial function and is liked to a host of mitochondrial functions, including ATP synthesis, import of mitochondrial proteins, calcium homeostasis and metabolite transport [13]. The results in this study showed that reduction in MMP induced by SNP is concentration dependent. Spermatozoa that exhibit high MMP generally have intact acrosome function and high fertilizing capacity as well as normal motility and morphology [14, 15].

Reduction in MMP is preceded by permeability transition pore (PTP) in the sperm. PTP is a multi-component protein that regulates oxidative phosphorylation, aggregates in mitochondrial membranes and induces cell death when it turns into a non-specific channel [15, 16, 17]. Another mechanism associated with low MMP is the coordination among numerous B-cell lymphoma (BCl-2) proteins to induce mitochondrial outer membrane permeabilization [18] and this mechanism is strictly associated with caspase activation [19, 20]. NO induces a dramatic increase in percentage of spermatozoa with caspase activity [21]. NO stimulates proteins of the BCl-2 family, followed by releasing of mitochondrial cytochrome C and concomitant activation of caspase 9 and 3 which results in disruption of MMP [22].

Conclusion

This study indicates that addition of SNP in the capacitation medium decreases sperm MMP in frozen thawed buffalo semen in concentration dependant manner.

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