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Effect of Nitric Oxide on Ram Sperm Motility *in vitro*

[1]Hossein Hassanpour, [2]Pejman Mirshokrai, [2]Abolfazl Shirazi and [1]Atefe Aminian
[1]Department of Basic Sciences, [2]Department of Clinical Sciences, College of Veterinary Medicine,
Sharekord University, Sharekord, Iran

Abstract: The aim of this study is to investigate the effects of NO on sperm motility of ram. After incubation of normozoospermic samples for 160 min in the presence of L-arginine (NOS substrate), SNP (NO donor), L-NAME (non-selective nitric oxide inhibitor) and packed erythrocytes (NO scavenger), sperm motility assessed in four grades (A, B, C, D). In this study, L-arginine and SNP non-significantly increased progressive motility at low concentration and significantly decreased progressive motility at high concentration. L-NAME and packed erythrocytes dose-dependently decreased progressive sperm motility. It is concluded that sperm motility of ram physiologically depend on nitric oxide action although excess generation of nitric oxide by sperm or exogenous NO could not increase sperm motility and it seems that excess NO provides toxic condition to decrease sperm motility.

Key words: Nitric oxide, ram, sperm motility, *in vitro*

INTRODUCTION

Nitric oxide (NO) is a gaseous free radical with short-half life that functions as a potent cell signaling, effector and vasodilator molecule in numerous tissues. NO is synthesized from a guanidino nitrogen atom of the essential amino acid L-arginine by a family of isoenzymes known as the nitric oxide synthases (NOS) (Pacher *et al*., 2007) in the presence of oxygen and the electron donors Nicotinic Acid Adenine Dinucleotide (NAD), Flavin Adenine Dinucleotide (FAD) or flavin mononucleotide (FMN) with L-citrulline occurring as a by-product. Three major NOS isoforms, which share a common basic structural organization and requirement for substrate cofactors for enzymatic activity, have been described. Endothelial (eNOS or NOS III) and neuronal (nNOS or NOSI) isoforms are constitutive, Ca^{2+}/calmodulin-activated and inducible isoform (iNOS or NOSII) is Ca^{2+-} independent and induced by many inflammatory factors such as cytokines and lipopolysaccharide (LPS) (Michel and Feron, 1997). Following the recognition of NO as a mediator of penile erection (Burnett, 2006), NOS protein and activity have been demonstrated both in male and female reproductive organs (McCann *et al*., 1999; Telfer *et al*., 1995), suggesting an involvement of NO in the physiology of reproduction. Evidence has been reported that NO can also be generated by spermatozoa. An immunoreactivity for NOS was observed in mouse, human (Herrero *et al*., 1996) and bull sperm (Meiser and Schulz, 2003).

It has been shown that human spermatozoa with normal motility, express significant eNOS protein while low motility spermatozoa exhibit aberrant patterns of eNOS expression (OBrayan *et al*., 1998). In semen collected from different subjects, a positive correlation was observed between the concentration of NO and percentage of immotile spermatozoa (Rosselli *et al*., 1995). The present study sought to investigate NO involvement in the motility of ram spermatozoa using activator, scavenger and inhibitor of NO or its enzymes.

MATERIALS AND METHODS

Chemicals: Synthetic oviductal fluid buffered with HEPES (SOF-HEPES) medium, L-arginine, Sodium nitroprusside (SNP) and NG-nitro-L-arginine methyl ester (L-NAME) were purchased from Sigma chemical company (St Louis, Mo, USA). Packed erythrocytes obtained from ram after removing of plasma and washing by saline.

Animals and management: The study was performed on Bakhtiari sheep in a farm of Agricultural Research Center (Shooli, Sharekord, Iran) at October and November. The rams were kept as a single flock in a semi-extensive production system. Bakhtiari rams were with mean weight of 95±5 kg and body condition scores (BCS) of 3.0-4.0 (scale 0-5).

Semen processing and preparation of sperm suspension: Semen was collected using an artificial vagina from twenty Bakhtiari rams. Generally one ejaculate per ram was

Corresponding Author: Hossein Hassanpour, Department of Basic Sciences, College of Veterinary Medicine, Sharekord University, Saman St., Sharekord, Iran Tel: +983814424427 Fax: +983814424427

collected on a daily basis. All ejaculates were evaluated using light microscopy within 30-45 minutes after collection (keeping in 37°C). Ejaculates were only used if the volume was ≥ 0.5 mL and had a sperm concentration ≥ 2×10^9 spermatozoa/mL and ≥ 60% motile cells. After measuring the sperm concentration (Neubauer chamber, 100 x magnifications) by light microscope, Motile spermatozoa were harvested by the swim-up technique (37°C for 1 h in air atmosphere) (Parrish *et al.*, 1986) using SOF-HEPES medium. The presence of round cells (spermatogonia, spermatocytes, spermatides and leucocytes) were minimal (less than 1×10^6 mL^{-1}) in all sperm samples after swim-up technique in final suspension. After swim-up, the motile sperm-rich fraction was centrifuged at 600 xg for 10 min at room temperature and after light microscopy evaluation again, sperm concentration was adjusted to approximately 5×10^8 cells mL^{-1} by adding SOF-HEPES medium. Aliquots of sperm suspension in SOF-HEPES medium, 500 μL each, containing 2×10^7 cells, were incubated for 120 min in presence of (0.1, 0.5, 0.7 μM) SNP, (1, 10, 100 μM) L-NAME, (0.1, 1, 10 mM) L-arginine and (1, 10 μL mL^{-1}) Packed erythrocytes separately and an equal volume of SOF-HEPES medium (controls). Then motility was evaluated by light microscopy according to WHO manual recommendation (WHO, 1999) at 37°C. Spermatozoa were graded: rapid progressive motility, grade A; slow or sluggish progressive motility, grade B; non-progressive motility, grade C; or immotility, grade D. At least 200 spermatozoa in five different microscopic fields were evaluated for each sample.

Statistical analysis: Results are expressed as mean±SEM that is obtained from at least 7 experiments in each group. The difference between experimental groups was tested by Student's t-test using SPSS-14 package. P-values less than 0.05 were considered statistically significant.

RESULTS

Effect of L-arginine in sperm motility: After incubation of sperm suspension by different concentrations of L-arginine, it was found that concentrations of 0.1 and 1 mM caused sperm motility grades A and C to be increased and grade D decreased, although these variations were not significant ($p<0.05$). Concentration of 10 mM conversely decreased grade A (and increased grade D significantly ($p<0.05$). Variations of this concentration in grade B and C were not significant (Fig. 1).

Effect of SNP in sperm motility: SNP in the concentration of 0.1 μM non-significantly increased sperm motility at grades A and C and decreased at grades B and D. Concentration of 0.5 μM increased grade B and D and decreased grade C. SNP at the concentration of 0.7 μM significantly decreased sperm motility at grade A (15.1%) and increased at grade D (16.7%), in comparison with their control groups ($p<0.05$). Decreasing of sperm motility at grades B and C at concentration of 0.7 μM were not significant (Fig. 2).

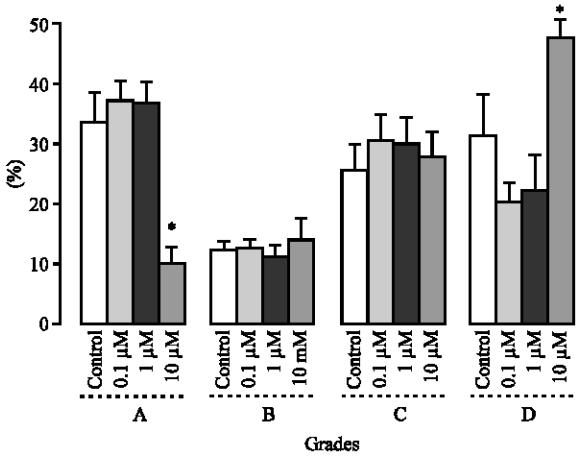

Fig. 1: Effect of L-arginine on sperm motility (A: fast progressive motility, B: slow progressive motility, C: non-progressive motility and D: immotility), in different concentrations. Values are means±SE. *$p<0.05$ from corresponding control

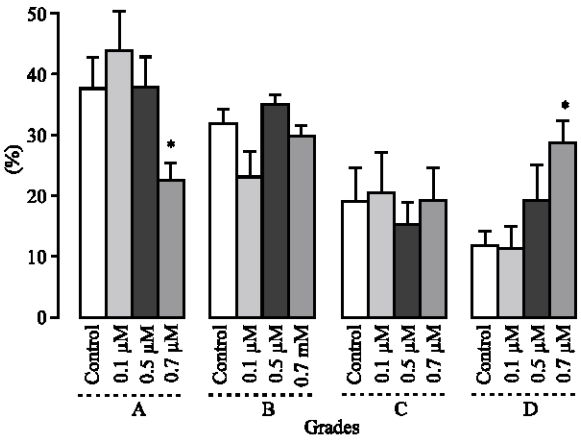

Fig. 2: Effect of SNP on sperm motility (A: fast progressive motility, B: slow progressive motility, C: non-progressive motility and D: immotility) in different concentrations. Values are means±SE. *$p<0.05$ from corresponding control

Effect of L-NAME in sperm motility: L-NAME at concentrations of 1 and 10 μM non-significantly decreased grade A and increased grade D. Variations in grade B and C were minimum. Concentration of 100 μM caused sperm motility grades A (15.3%) significantly ($p<0.05$) and B non-significantly to be decreased and grade C non-significantly and D (10.2%) significantly increased in compared with their controls ($p<0.05$) (Fig. 3).

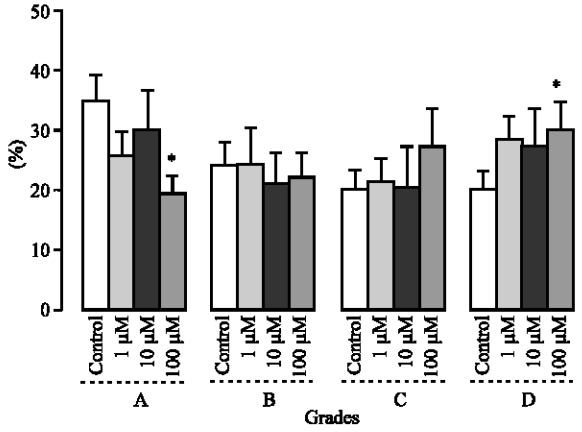

Fig. 3: Effect of L-NAME on sperm motility (A: fast progressive motility, B: slow, progressive motility, C: non-progressive motility and D: immotility) in different concentrations. Values are means±SE. *$p<0.05$ from corresponding control

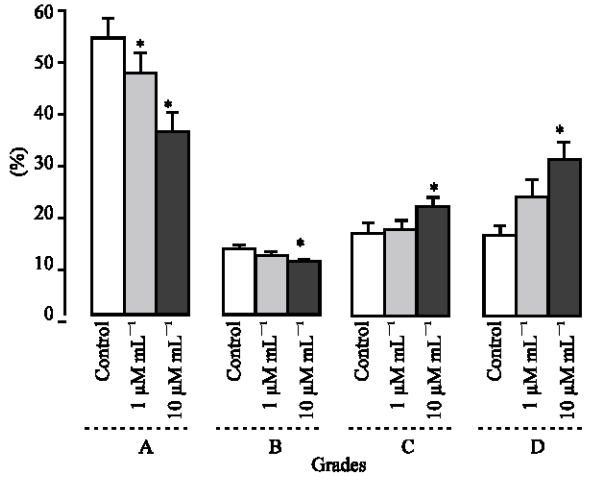

Fig. 4: Effect of packed erythrocytes on sperm motility (A: fast progressive motility, B: slow progressive motility, C: non-progressive motility and D: immotility) in different, concentrations. Values are means±SE. *$p<0.05$ from corresponding control

Effect of packed erythrocytes on sperm motility: Packed erythrocytes at concentration of 1 μL mL^{-1} significantly ($p<0.05$) decreased grade A (7.1%) and non-significantly increased grade D. Variations in grade B and C were minimum. Concentration of 10 μL mL^{-1} showed grades A (18.2%) and B (2.5%) to be reduced and grades C (5.4%) and D (15%) enhanced. These variations were significant when compared to their control groups ($p<0.05$) (Fig. 4).

DISCUSSION

NO is a molecule of great biological significance and has long been considered to play an important role in sperm physiology such as sperm chemotaxis, sperm motility, sperm-egg interaction and spermatogenesis (Revelli *et al*., 2002). Many studies suggest that spermatozoa could represent one of the targets of NO, and a role for NO in controlling the function of male gametes during their maturation and migration in the genital tracts is conceivable. In fact, human and rodent sperm have been reported to be influenced by exogenous NO, as far as motility, viability and metabolism are concerned (Herrero *et al*., 1994; Hellstrom *et al*., 1994; Weinberg *et al*., 1995). NO could even participate in the mechanisms leading to fertilization, as in bovine sperm it increases capacitation and acrosomal reactivity (Zamir *et al*., 1995).

In this study, at the first it is found that effects of L-arginine on sperm motility are dose- dependent in vitro, and low concentrations of L- arginine increasingly affect on sperm motility, while high concentration of L-arginine conversely decrease. Srivastava *et al*. (2006) determined that NO is synthesized from L-arginine by the enzyme Nitric Oxide-Synthase (NOS) present in spermatozoa, and they suggested a possible participation of NO and NOS in arginine action. *In vitro* studies have shown that low concentrations of NO enhance the motility of mouse (Herrero *et al*., 1994) and human (Zhang and Zheng, 1996) spermatozoa and medium/high concentrations of NO decreasing sperm motility (Rosselli *et al*., 1995). It is tempting to speculate in our condition, small amounts of NO are generated by L-arginine that neutralizes free radicals which inhibit sperm motility. In contrast, excessive generation of NO by high concentration of L-arginine can cause sperm toxicity as well as reduce sperm motility by contributing to the formation of peroxynitrite, a highly toxic anion of peroxidation (Levonen *et al*., 2001). Ratnasooriya and Dharmasiri (2001) also demonstrated that L-arginine caused a dose-dependent impairment of hyperactivated motility *in vitro*, which can obviously inhibit fertilizing potential of sperm. However, this data confirm present findings.

Present results showed that SNP as a No donor, could affect sperm motility increase at low concentration. In contrast, its high concentration declined sperm motility, these findings as similar as Rosselli *et al.* (1998), Nobunaga *et al.* (1996) and Weinberg *et al.* (1995) results, also strongly suggest that exogenous NO is beneficial at low concentration for ram sperm motility and is harmful at high concentration.

At this study, L-NAME as nitric oxide synthase inhibitor and packed erythrocytes as nitric oxide scavenger dose-dependently decreased progressive motility and increased immotile sperm. These data provide evidence that motility of ram spermatozoa is regulated autocrinally by nitric oxide. A study by Lewis *et al.* (1996) also suggested the presence of eNOS and nNOS in the human spermatozoon that regulates (increases) sperm motility in an autocrine fashion and they demonstrated that, as compared to normozoospermic samples, the expression of eNOS and concentrations of NO generated were lower in asthenozoospermic samples, suggesting that decreased endogenous NO may influence sperm motility and hence fertilization.

Taken together, the present study showed that sperm motility of ram physiologically is affected nitric oxide action although excess generation of nitric oxide by sperm or exogenous NO could not increase sperm motility and it seems that excess NO provide toxic condition to decrease sperm motility.

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