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Possible Role of Nitric Oxide on Fertile and Asthenozoospermic Infertile Human Sperm Functions*

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The capacity of human sperm fertilization is principally dependent on sperm motility and membrane integrity. Oxygen-derived free radicals, such as superoxide anion, are known to impair sperm motility and membrane integrity by inducing membrane lipid peroxidation (LPO). Nitric oxide (NO), a biologically active free radical, has recently been shown to inactivate superoxide and increase intracellular guanosine-3', 5'-cyclic monophosphate (cGMP). The aim of this study is to investigate the effects of NO on human sperm motility, viability, lipid peroxidation and cGMP in fertile and asthenozoospermic infertile individuals *in vitro*. Semen samples were obtained from 10 fertile volunteers and 10 asthenozoospermic infertile patients. Washed spermatozoa were incubated at 37°C in Ham's F-10 medium with 0, 25, 50, 100, 200, or 400nM sodium nitroprusside (SNP, Na_2 [Fe(CN) $_5$NO] · $2H_2O$), a nitric oxide releaser. Samples were analyzed for viability, determined by eosin-Y dye exclusion method at 0, 1, 2, 3, 5 and 6 h of incubation; motility, determined by the trans-membrane migration method within 2 h of incubation; LPO determined by malondialdehyde (MDA) -thiobarbituric acid method at 3 h of incubation; and the intracellular cGMP, determined by ^{125}I-cGMP radioimmunoassay at 3 h of incubation. The results showed: in both fertile and infertile samples, viability, trans-membrane migration ratio and the levels of intracellular cGMP in 25–100nM SNP-treated spermatozoa were significantly higher than those in control groups, while MDA contents in treated groups were significantly lower than those in controls. However, when concentrations of SNP increased to 200–400nM, the opposite effects were exhibited. The effects of SNP on these processes were biphasic within 25–400nM. The most effective concentration was 100nM. These data suggested that NO is beneficial to sperm viability and motility in both fertile and infertile individuals, and that reduction of lipid peroxidative damage to sperm membranes and increase of intracellular cGMP may be involved in these benefits.

Keywords: Nitric oxide, sodium nitroprusside, sperm motility, sperm viability, lipid peroxidation, cGMP

INTRODUCTION

The prognosis for infertile couples has improved dramatically in recent years with the application and improvement of various techniques of diagnosis and treatment. However, for male-factor infertility induced by defective sperm function, the ability to provide effective treatment remains poor. Poor sperm motility rather than a low total

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sperm count or abnormal morphology is considered to be the main cause of male infertility.[1] The motility of spermatozoa which develops during epididymal maturation is required in fertilization for transport to the egg and penetration of the zona pellucida. Moreover, motility pattern was shown to be closely correlated with natural pregnancy rate[2] as well as with *in vitro* fertilization rate.[3] The fusion of sperm and egg membranes is also one of the important functions of spermatozoa to complete fertilization, which requires high integrity and fluidity of membranes. Fertilization will be impaired if either membrane is damaged.[4] Human spermatozoa can generate reactive oxygen species (ROS), such as superoxide anion (O_2^-), and are especially sensitive to oxidative damage because of their high concentration of polyunsaturated fatty acids and their relatively low levels of antioxidant enzymes.[5–7] The effects of ROS on sperm plasma membranes are thought to be responsible for loss of sperm motility and other sperm functions.[5–7]

Nitric oxide (NO) is a biologically active free radical which is generated in mammalian cells from L-arginine by nitric oxide synthase (NOS).[8,9] NOS, a Ca^{2+}/calmodulin-dependent enzyme, has been isolated from rat testis, epididymis, seminal vesicle, vas deferens[10] and human pelvic and genital structures[11] besides being localized to nerve cells.[12] Recent studies have shown that NO could maintain post-thaw human sperm motility and viability, and reduce lipid peroxidative damage to sperm membranes,[12] by its ability to inactivate O_2^-.[8] Due to its potent effect of vasodilation, NO can result in cavernosal smooth muscle relaxation and, eventually to result in penil erection.[14,15] NOS inhibition could completely prevent physiological ejaculation, and the drugs that act as a NO donor could treat ejaculatory incompetence.[15] Promotion of hamster sperm hyperactivation and capacitation by NO were also observed in other experiments,[16,17] which was thought to involve the activation of guanylate cyclase and formation of guanosine-3′, 5′-cyclic monophosphate (cGMP).[16,17] cGMP is an important second messenger in cells related to cellular metabolic activity by activating adenosine-3′, 5′-cyclic monophosphate (cAMP)—dependent protein kinases as well as cGMP-dependent protein kinases.[18] Increased cGMP levels have been shown to increase sperm metabolism and motility in the sea urchin and other nonmammalian species,[19,20] thus, we speculate that cGMP may also affect mammalian sperm motility.

The aim of the present study is to investigate the effect of NO *in vitro* on human sperm motility, viability and lipid peroxidation in fertile and asthenozoospermic infertile individuals and the mechanism of these effects, so as to evaluate the possibility of treating asthenozoospermic infertility by NO. Because NO has a very short half life and is degraded easily, sodium nitroprusside (SNP), which can spontaneously relase NO without enzymatic conditions,[21] was usually used as a NO doner.[9,13,22,23]

MATERIALS AND METHODS

Materials

Ham's F-10 culture medium was purchased from Sigma Chemical Co. (St Louis, MO, USA). Sodium nitroprusside was purchased from Beijing Pharmaceutical Industrial Institute (Beijing, China). Nucleopore filter membranes were purchased from Shanghai Nuclear Institute, Academy of Science, China (Shanghai, China). ^{125}I-cGMP radioimmunoassay medical kit (response 10^{-15}mol, optimal test range 0.01–0.4pmol) was provided by Department of Isotope, Shanghai Second University of Medicine (Shanghai, China). All other chemicals were purchased from Shanghai Biochemical Co. (Shanghai, China) and were reagent grade.

Collection, Preparation and Treatment of Semen Samples

Normal semen samples were obtained from 10 fertile volunteers (25–35 years old) who were

working and studying in Lanzhou Medical College, with the sperm characteristics: >30 × 10^6 spermatozoa ml^{-1}, >60% viable, >50% rapid and linear progressive motility and >60% normal sperm morphology. The asthenozoospermic samples came from 10 infertile patients (27–40 years old) attending the Male Infertile Clinic of Lanzhou Medical College, who had <40% motility and the other semen parameters were otherwise similar to those of normal samples. They had no past or present history of systemic diseases and had received no drugs during the 6 months prior to the study. The female partners of these men were normal with respect to tubal patency and menstrual cycle. Semen samples were collected into sterile containers by masturbation after 5–7 days of sexual abstinence. After allowing 30 min at room temperature for liquefaction to occur, the spermatozoa were washed and resuspended in Ham's F-10 culture medium (pH 8). The sperm concentration in the suspension was adjusted to 20 × 10^6 spermatozoa ml^{-1}. Sperm suspensions were then divided into six equal aliquots, which were incubated respectively with 0, 25, 50, 100, 200 or 400nM SNP (final concentrations) at 37°C in 5% CO_2.

Determination of Sperm Viability

The percentage of viable sperm presented in each aliquot was assessed respectively at 0, 1, 2, 3, 5 and 6 h after SNP was added. A 7 µl sperm suspension of each aliquot was mixed with an equal volume of 0.05% eosin-Y. After a 2 min incubation at 37°C, slides were viewed with a bright-field microscope at 400 × magnification. Dead spermatozoa appear pink and live spermatozoa are not stained. Two hundred spermatozoa were counted for each sample and viability percentages calculated.

Determination of Sperm Motility

Sperm motion was evaluated by the trans-membrane migration method.[24] A 100 µl sample of each aliquot was pipetted into a glass ring with 10 mm diameter. The lower end of the ring was covered with a nucleopore filter membrane in which there are evenly distributed 5 µm capillary pores. The ring was inserted and fixed tightly into a glass bottle containing 2 ml of Ham's F-10 medium, and was incubated for 2 h at 37°C in 5% CO_2. The ring was then removed. Spermatozoa which penetrated through the filter membrane into the medium in the glass bottle were killed with 50µl of formalin. The number of spermatozoa in the glass bottle was counted and the trans-membrane migration ratio (TMMR) was calculated by the proportion of spermatozoa in the ring and in the glass bottle.

Determination of Lipid Peroxidation

Sperm membrane lipid peroxidation was monitored using the generation of malondialdehyde (MDA), a product of lipid peroxidation. MDA was assessed by the thiobarbituric acid (TBA) method[13] at 3 h of incubation. An aliquot of each group was incubated with 12.7 mM ascorbic acid and 2.3 mM ferrous sulfate (final concentrations) for 1 h in a 37°C shaking water bath, and then ice-cold 2.4 M trichloroacetic acid (final concentration) was added to precipitate protein. The sample was then centrifuged at 3500 × g for 25 min, 4°C. TBA 1% in 12.5 mM NaOH (final concentration) 0.3 ml was add into the 1.7 ml supernatant. This solution was boiled for 30 min in a water bath. Once the tubes had cooled to room temperature, the absorbance at 535nm was measured and then was compared with MDA standard curve. The content of MDA was expressed as nM/10^8 spermatozoa. This determination was repeatedly performed in 10 replicates (n = 10) for each sample and the coefficient of variation was 16.29%.

Determination of cGMP in Sperm

After incubation for 3 h, 1N $HClO_4$ 0.5ml was added into 1 ml sperm suspension from each aliquot. Spermatozoa were intermittently homo-

genized in the ice-water bath for 3 min with ultrasonic homogenizer (Jc-2 model, Tong Hua Ultrasonic Equipment Factory, China) and then were centrifuged 1200 × g for 20 min at 4°C. The supernatant was adjusted to pH 7.0 with 2M KOH, and was centrifugated again. The supernatant was used to determine the intracellular cGMP with ^{125}I-cGMP radioimmunoassay.[25] Acetyl reagent 8 µl, ^{125}I-cGMP 50 µl (1000 cpm/50µl) and cGMP antiserum (1:3000) were successively added to a tube containing 100µl of the acetale buffer solution and 100µl of the testing supernatant. All reactions were performed in ice-water bath. The tube was put in 0–4°C to react for 16–24 h. Combined and free cGMP were successively separated by filtering. The radioactivity of each tube was measured with γ -ray counter (FJ-2008 model, Xi'an Nuclear Instrument Factory, China). The concentration of cGMP was calculated according to the standard curve. The cGMP content was expressed as pmol/10^8 spermatozoa. This determination was repeatedly performed in 10 replicates (n = 10) for each sample and the coefficient of variation was 45.12%.

Statistical Analysis

Each value is expressed as the mean and standard error of mean(SEM). The differences among data of individual groups were assessed using analysis of variance (ANOVA), and then the data of testing groups were compared with the data of controls by Dunnet's method. A P value less than 0.05 was selected as the criterion for statistically significant differences.

RESULTS

Effect of SNP on Sperm Viability

In control groups, sperm viability in both fertile and infertile samples significantly declined with incubation time from 75.2 ± 11.3% and 61.3 ± 4.1% at 0 h to 62.1 ± 10.4% and 23.2 ± 6.1% at 6 h, respectively. The decline in sperm viability was profound in infertile samples compared to the controls (Fig. 1). Compared with controls, sperm viability in fertile and infertile samples increased with concentrations of SNP from 25, 50 and up to 100nM, and then decreased with concentrations of SNP from 200 to 400nM (Fig. 1). Thus, the 100nM of SNP was considered to be the optimal mantaining concentration for sperm viability over time. The viability in the control group was lower in the infertile group compared to the fertile samples.

Effect of SNP on Sperm Motility

The trans-membrane migrations of sperm were significantly improved by SNP up to 100nM in

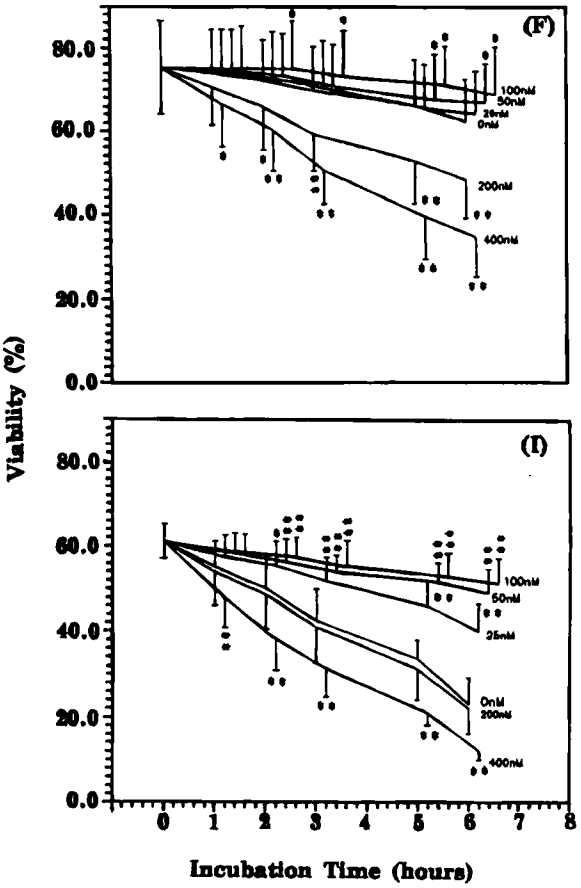

FIGURE 1 Effects of different concentrations of sodium nitroprusside on fertil (F) and asthenozoospermic infertile (I) human sperm viability at 0, 1, 2, 3, 5 and 6 h. Data represent mean ± SEM, n = 10, *: P < 0.05; **: P < 0.01 *vs* control.

both fertile and infertile groups. Although sperm motility was significantly enhanced by SNP at a concentration of 200nM, the motility was less than that achieved with 100nM of SNP. SNP at a concentration of 400nM induced a significant reduction in sperm motility. Consequently, the optimal concentration of SNP on sperm motility was 100nM (Fig. 2).

Effect of SNP on Sperm Lipid Peroxidation

MDA content in infertile sperm is significantly higher than that in fertile sperm. Compared with controls, MDA contents were significantly reduced with increasing concentrations of SNP up to 100nM and then recovered at 200 and 400nM in both fertile and infertile samples. Maximal reduction in MDA content was observed with 100nM SNP (Fig. 3).

Effect of SNP on Sperm cGMP Level

The levels of intracellular cGMP in both fertile and infertile spermatozoa treated with SNP were significantly increased with increasing concentrations of SNP up to 100nM and then decreased thereafter. The most effective concentration of SNP is 100nM (Fig. 4).

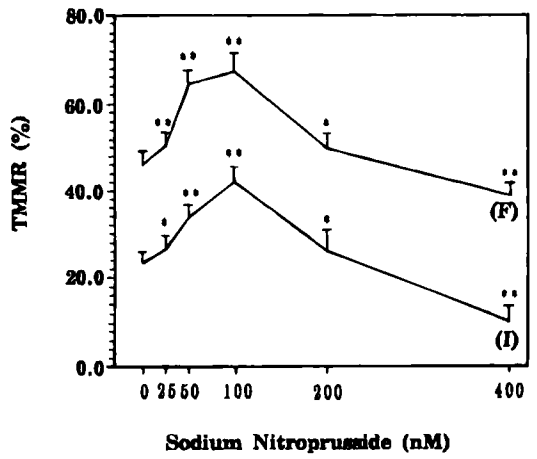

FIGURE 2 Effects of different concentrations of sodium nitroprusside on fertile (F) and asthenozoospermic infertile (I) human sperm motility at 2 h. Data represent mean ± SEM, n = 10, *: $P < 0.05$; **: $P < 0.01$ *vs* control.

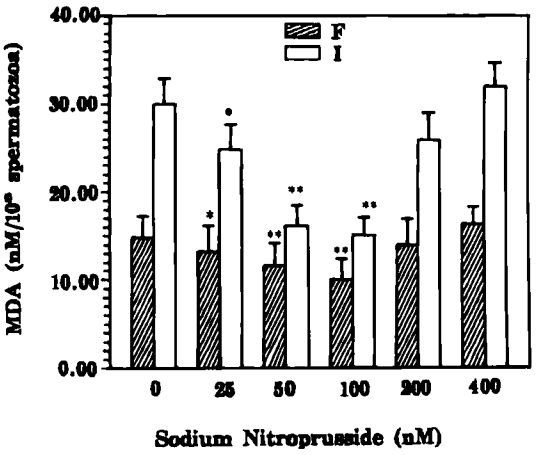

FIGURE 3 Effects of different concentrations of sdoium nitroprusside on fertile (F) and asthenozoospermic infertile (I) human sperm lipid peroxidation following 3 h of incubation at 37°C in 5% CO_2. Data represent mean ± SEM, n = 10, *: $P < 0.05$; **: $P < 0.01$ *vs* control.

DISCUSSION

The effects of SNP on sperm viability, motility, membrane lipid peroxidation and intracellular cGMP were biphasic within the range of 25–400nM. The first phase occured within 25–100nM for increasing viability, motility and cGMP and decreasing MDA content, and these second phase occured at 200 and 400nM and acted the in opposite direction, i.e. decreasing viability, motility and cGMP and increasing MDA content. The maximally effective concentration of SNP for all 4 parameters tested is 100nM.

NO is employed in a host of biological processes as an important messenger molecule, it functions as an atypical neurotransmitter in the central nervous system,[26] as an agent of non-adrenergic, non-cholinergic autonomic neurotransmission in the pheriphery,[27] as an important factor in leukocyte adhesion to venules,[28] an effective inhibitor of platelet aggregation[8] in the circulatory system, and as

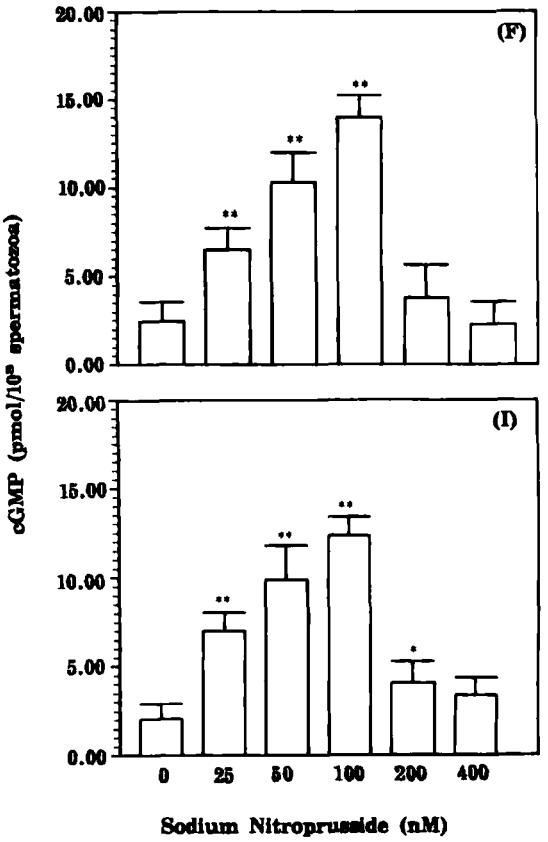

FIGURE 4 Effects of different concentrations of sodium nitroprusside on fertile (F) and asthenozoospermic infertile (I) human sperm cGMP following 3 h of incubation at 37°C in 5% CO_2. Data represent mean ± SEM, n = 10, *: $P < 0.05$; **: $P < 0.01$ *vs* control.

a major active substance in the cytotoxic function of neutrophils in the immune system.[8] Recently, NO has been shown to play a significant role in reproductive functions.[13,17] The presence of NOS in the adrenal and pituitary glands, in the testis and epididymis, and the effect of NOS inhibitor in these tissues to testosterone production[29] also support the speculation of NO playing an important role in modulation of sperm functions. The mechanism by which NO exerts its effects is thought to be by activation of soluble guanylyl cyclase which accelerates the production of cGMP.[9,12,14,30] The present study has also shown that sperm intracellular cGMP was significantly increased and sperm motility was also enhanced after samples were incubated with SNP as a non-enzymatic source of NO. It is known that cGMP increases cellular metabolic activity by activating cGMP-dependent protein kinase and cAMP-dependent protein kinase or by activating a membrane-bound Ca^{2+}-ATPase which promotes calcium sequestration inside the mitochondria.[31] The energy for supporting sperm motion mainly derives from adenosine triphosphate metabolism.[32] cGMP can increase the sperm metabolic rate and energy generation, which results in enhancement of sperm motility.[19,20]

In the present study, sperm viability in both fertile and asthenozoospermic infertile samples were significantly reduced with incubating time, especially in asthenozoospermic infertile spermatozoa. Whereas sperm viability was maintained for up to 6 h and sperm motility was significant improved in samples incubated with SNP. It is well known that human sperm fertilizing capacity is principally dependent on sperm motility and sperm membrane integrity, and thus fertilization will be impaired if they are damaged. Human spermatozoa are especially sensitive to oxidation, and their motility and other functions would be inhibited by ROS.[5–7] The present experiment also showed that the level of lipid peroxidation in asthenozoospemic infertile spermatozoa was significantly higher than that in fertile spermatozoa. It indicated that spermatozoa from infertile men are more sensitive to oxidative stress than sperm from fertile men. In addition to stimulation of guanylate cyclase the other important biological function of NO is to protect cells from peroxidative damage as a free radical scavenger by inactivating O_2^-.[8] However, on the other hand, O_2^- can inactivate NO in turn.[8] The addition of exogenous NO increased the ratio of NO/O_2, and resulted in superoxide inactivation.[13] Although the mechanism of biphasic actions of SNP on sperm is still unknown, SNP is always used for treatment of hypertension as a nitrovasodilator and it is safe below an *iv gtt* dose of 3mg/kg.[33]

However, SNP is also a cyanide like compound which can damage cells by inhibiting respiratory enzymes of the mitochondrion when its anount is excessive. Moreover, excess of NO itself in tissues has also been shown to result in cell damage even death,[34] which may be involved in the formation of toxic peroxynitrite in the reaction of NO with O_2^-.[35]

We suggested that NO induced enhancement of sperm viability and motility may be mediated by the inhibition of sperm membrane lipid peroxidation and the enhancement of sperm cGMP generation. NO treatment of semen may have application in the treatment of asthenozoospermic infertility.

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