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Ginsenoside R_e Increases Fertile and Asthenozoospermic Infertile Human Sperm Motility by Induction of Nitric Oxide Synthase

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We investigated the effects of Ginsenoside R_e on human sperm motility in fertile and asthenozoospermic infertile individuals *in vitro* and the mechanism by which the Ginsenosides play their roles. The semen samples were obtained from 10 fertile volunteers and 10 asthenozoospermic infertile patients. Spermatozoa were separated by Percoll and incubated with 0, 1, 10 or 100 μM of Ginsenoside R_e. Total sperm motility and progressive motility were measured by computer-aided sperm analyzer (CASA). Nitric oxide synthase (NOS) activity was determined by the ^{3}H-arginine to ^{3}H-citrulline conversion assay, and the NOS protein was examined by the Western blot analysis. The production of sperm nitric oxide (NO) was detected using the Griess reaction. The results showed that Ginsenoside R_e significantly enhanced both fertile and infertile sperm motility, NOS activity and NO production in a concentration-dependent manner. Sodium nitroprusside (SNP, 100 nM), a NO donor, mimicked the effects of Ginsenoside R_e. And pretreatment with a NOS inhibitor N^{ω}-Nitro-L-arginine methyl ester (L-NAME, 100 μM) or a NO scavenger *N*-Acetyl-L-cysteine (LNAC, 1 mM) completely blocked the effects of Ginsenoside R_e. Data suggested that Ginsenoside R_e is beneficial to sperm motility, and that induction of NOS to increase NO production may be involved in this benefit.

Key words: Ginsenoside R_e, Sperm motility, Nitric oxide synthase, Nitric oxide, Reactive oxygen species

INTRODUCTION

The motility is an important function of normal mature spermatozoa, and is required during fertilization for transport to the egg and penetration of the zona pellucide. Poor sperm motility rather than a low total sperm count or increased numbers of spermatozoa with abnormal morphology is considered to be more likely reason for male infertility (Cai and Marik, 1989; Yunes *et al.*, 2003).

Nitric oxide (NO) is a biological active free radical and also an important intracellular and intercellular messenger which is generated in mammalian cells from L-arginine by a family of nitric oxide synthases (NOS) (Marletta, 1993). Both neuronal NOS (nNOS), first localized in neurons, and endothelial NOS (eNOS), first identified in endothelial cells, are constitutive Ca^{2+}/ calmodulin-dependent, whereas the macrophagic NOS (iNOS) is an inducible Ca^{2+}/ calmodulin-dependent isoform (Moncada *et al.*, 1991; Forstermann *et al.*, 1994). NOS protein and activity have been detected in human, rat and mouse testis, human and mouse spermatozoa, and human and rat epididymis, prostate and seminal vesicles (Ehren *et al.*, 1994; Burnett *et al.*, 1995; Lewis *et al.*, 1996; Herrero *et al.*, 1997; O'Bryan *et al.*, 1998; Revelli *et al.*, 1999; Kon *et al.*, 2002; Balercia *et al.*, 2004). NO, besides being involved in multiple biological processes in neurotransmission, regulations of vascular wall tone and immune system activity, has recently been indicated to play a significant role in modulation of sperm functions. Low concentrations of exogenous NO donors have been shown to enhance post-thaw and asthenozoospermic human sperm motility and viability (Hellstrom *et al.*, 1994; Zhang and Zheng, 1996), human sperm capacitation (Zini *et al.*, 1995; Herrero

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et al., 1999), hamster sperm hyperactivation (Yeoman *et al.*, 1998), and human sperm binding to the zona pellucida (Sengoku *et al.*, 1998). Moreover, evidences have been found that NO can also be generated by spermatozoa and this endogenous NO is beneficial to sperm motility (Lewis *et al.*, 1996) and acrosomal reaction (Revelli *et al.*, 1999).

Ginsenosides are the biologically effective components of ginseng and are widely used in traditional Chinese medicine to enhance stamina and capacity to cope with fatigue as well as physical stress. They have been reported to scavenge free radicals (Chen, 1996) and protect against myocardial ischaemia/reperfusion damage (Chu and Chen, 1990). And studied have also found that Ginsenosides enhanced NO production in cultured porcine endothelial cells (Li *et al.*, 2000), rat ventricular myocytes (Scott *et al.*, 2001), rat thoracic aorta (Kim *et al.*, 2003) and guinea-pig cardiomyocytes (Bai *et al.*, 2004) by inducing NOS activity. Recently, Ginsenosides have been shown to increase human sperm motility *in vitro* (Chen *et al.*, 1998, 1999, 2001). However, the mechanism involved in this effect of Ginsenosides is unknown.

Among 30 different ginsenosides, ginsenoside R_e is the major ingredient of *Panax ginseng* (Gillis, 1997) and has been widely reported to stimulate the activity of NOS in a variety of cells and tissues (Jin and Liu, 1994; Kang *et al.*, 1995; Jin, 1996; Scott *et al.*, 2001; Bai, 2003, 2004). The aim of the present study, therefore, is to investigate the effects of Ginsenoside R_e on human sperm motility in fertile and asthenozoospermic infertile individuals *in vitro* and the mechanism by which the Ginsenosides play their roles so as to evaluate the possibility for treating asthenozoospermic infertility by Ginsenosides. We revealed for the first time that Ginsenoside R_e improved human sperm motility by inducing NOS activity to increase endogenous NO production.

MATERIALS AND METHODS

Materials

Ginsenoside R_e ($C_{48}H_{82}O_{18}$), *N*-acetyl-L-cysteine (LNAC), N^{ω}-nitro-L-arginine methyl ester (L-NAME), Sodium nitroprusside (SNP), NADPH, Dowex 50WX8 (Na^+ form), Percoll and Ham's F-10 culture medium were purchased from Sigma Chemical Co. (St Louis, MO). L-[2, 3, 4-^{3}H]-Arginine monohydrochloride (specific activity 45-70 Ci/mmol; 1.0 mCi/mL) was obtained from PerkinElmer Life Sciences (Boston, MA). Biocinchoninic acid (BCA) protein assay kit was from Pierce Biotechnology, Inc. (Rochford, IL). WesternBreeze Chemiluminescent Immunodetection Kit was from Invitrogen Co. (Carlsbad, CA). Macrophagic NOS (iNOS) and endothelial isoform of constitutive NOS (ecNOS) rabbit polyclonal antibodies, and anti-β-actin antibody were purchased from Santa Cruz Biotechnology, Inc.(Santa Cruz, CA). All other chemicals were purchased from Shanghai Sangon Co. (Shanghai, China) and were reagent grade.

Collection, preparation and treatment of semen samples

Normal semen samples were obtained from fertile volunteers (25-35 years old) with the sperm characteristics: >30×10^6 spermatozoa mL^{-1} >60% viability, >50% rapid and linear progressive motility and >60% normal sperm morphology. The asthenozoospermic samples came from infertile patients (27-35 years old) attending the Male Infertile Clinic of Lanzhou Medical College, who had <40% sperm motility and the other semen parameters were similar to those found in 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 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 and allowed 30 min at room temperature for liquefaction to occur. The spermatozoa separated from seminal plasma by Percoll (65-95%) density gradient centrifugation were washed (200×g for 8 min) and resuspended in Ham's F-10 culture medium (supplemented with 10 mM Hepes and adjusted to pH 8). The sperm concentration in the suspension was adjusted to 20×10^6 spermatozoa mL^{-1}. Sperm suspensions were incubated respectively with 0, 1, 10, 100 μM of Ginsenoside R_e dissolved in deionized water or a NO donor sodium nitroprusside (SNP, 100 nM) at 37°C in 5% CO_2. In some groups, a NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME, 100 μM) or a NO scavenger *N*-acetyl-L-cysteine (LNAC, 1 mM) was incubated with the sperm suspensions for 15 min before R_e (100 μM) addition.

Determination of sperm motility

At 2 h after incubation, total sperm motility and progressive motility (spermatozoa which exhibit an actual space-gain motility) were measured by computer-aided sperm analyzer (CASA) (SQIAS-1000S, Guangzhou Longest Science & Technology Co., LtD, Guangzhou, China), according to the manufacturer's protocols.

Measurement of NOS activity in spermatozoa

NOS activity in sperm was measured as described by Revelli *et al.* (1999). After incubation for 2 h, 1 mL sperm suspension (20×10^6 spermatozoa mL^{-1}) from each aliquot was centrifuged at 12,000×*g* for 30 s, washed with 1×PBS, and then resuspended in Hepes/EDTA/DTT buffer (20 mM Hepes, 0.5 mM EDTA and 1 mM DTT, pH 7.2). The suspension was lysed by adding 0.1% Triton X-100, the following reagents were added to 100 μL cell lysate up to

a final volume of 120 μL: 2 mM NADPH, 1.5 mM $CaCl_2$, 2.5 μCi L-[^{3}H]-arginine (final concentration). A blank was prepared by replacing the cell lysate with an equal volume of Hepes/EDTA/DTT buffer. After a 15 min incubation at 37°C, the reaction was stopped by adding 2 mL cold Hepes-Na/EDTA buffer; the whole reaction mixture was applied to 2 mL columns of Dowex 50WX8 (Na^+ form) and eluted with 4 mL of water. The radioactivity corresponding to [^{3}H] citrulline content in about 6.1 mL eluate was measured by liquid scintillation. NOS activity was expressed as pmol of citrulline produced/minute/mg of cell protein. The protein concentration of samples was determined by the BCA protein assay kit.

Western blot analysis

After incubation for 2 h, 1 mL sperm suspension (20×10^6 spermatozoa mL^{-1}) from each aliquot was centrifuged at 12,000×*g* for 30 s and washed with 1×PBS. Protein extracts were prepared by lysing sperm with a solubilization buffer [62.5 mM Tris–HCl, 10% glycerol, 1% sodium dodecyl sulfate (SDS), and 4% protease inhibitor cocktails (10 mg/mL aprotinine, 10 mg/mL leupeptin, 10 mg/mL soybean trypsin inhibitor and 1 mM phenylmethylsulfonyl fluoride), pH 6.8]. The suspension was vigorously vortexed for 4 min, centrifuged at 10,000×*g* for 10 min at 4°C and the supernatant containing solubilized proteins was collected. The protein concentration of samples was determined by the BCA protein assay kit. 40 μg of each protein sample was exposed to reducing conditions (heated at 99°C for 4 min in the presence of 100 mM dithiothreitol), subjected to 8% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and then transferred to nitrocellulose membrane according to standard protocols. Western blotting was performed with the WesternBreeze Chemiluminescent Immunodetection Kit according to the manufacturer's protocol. In brief, the membrane was blocked for 30 min at room temperature and then probed with iNOS and ecNOS rabbit polyclonal antibodies diluted 1:200 (according to the recommendation of manufacturer) in blocking solution. The membranes were washed, and then the secondary antibody, AP-conjugated anti-rabbit IgG, was applied. Reprobing the blot with anti-actin antibody was performed to verify that the amount of protein in each well of the gel was identical. The protein was visualized using the chemiluminescence substrate provided with the kit.

Assay of NO production

The production of sperm NO (shown by the accumulation of nitrite, the stable end–product of the NOS/NO pathway) was detected according to the method of Griess (Green *et al.*, 1982). Briefly, after incubation for 2 h, 1 mL sperm suspension (20×10^6 spermatozoa mL^{-1}) from each aliquot was sonicated and then centrifuged at 1500×*g* for 15 min. The supernatant was mixed with equal volumes of Griess reagent (1% sulfanilamide, 0.1% *N*-1-naphthylenediamine dihydrochloride and 2.5% phosphoric acid), absorbance was measured at 540 nm after incubation for 10 min at 37°C in the dark. Nitrite concentration was expressed as nmol/10^6 sperm. These determinations were repeatedly performed using 10 replicates (n = 10).

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 the analysis of variance (ANOVA) (unpaired values). Then, the data of the testing groups were compared with the data of controls by Dunnet's method. A *p*-value less than .05 was selected as a criterion for a statistically significant difference.

RESULTS

Ginsenoside R_e increases sperm motility

Total sperm motility and progressive motility were significantly enhanced in both fertile and infertile samples in which Ginsenoside R_e was added. This effect was dose dependent and more important for sperm samples from

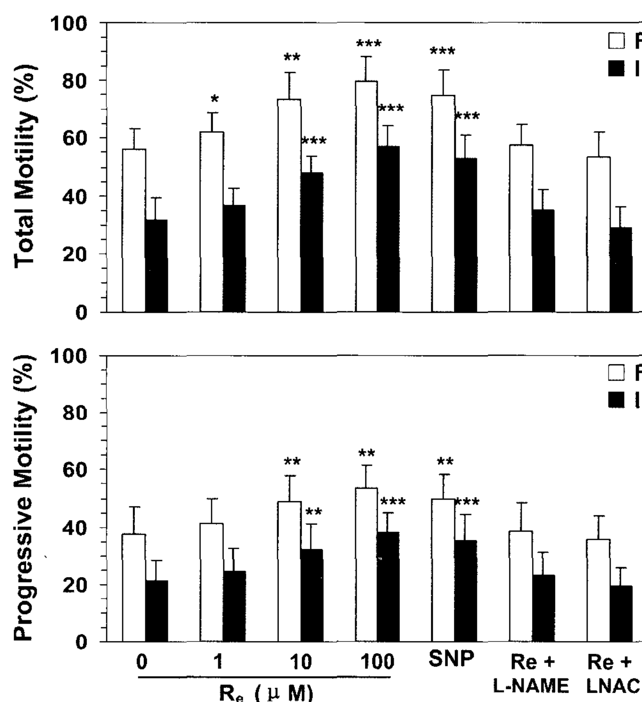

Fig. 1. Effects of different concentrations of Ginsenoside R_e, SPN (NO donor), or L-NAME (NOS inhibitor) and LNAC (NO scavenger) on fertile (□ F) and asthenozoospermic infertile (■ I) human total sperm motility and progressive motility at 2 h. Data represent mean ± SEM, n = 10, *p < .05; **p < .01, ***p < .001 vs. control.

infertile men than for those of fertile men (Fig. 1). 100 nM of SNP (a NO donor) mimicked the effects of Ginsenoside R_e, and a NOS inhibitor (L-NAME, 100 μM) or a NO scavenger (LNAC, 1mM) completely blocked the effects of Ginsenoside R_e, respectively (Fig. 1).

Effect of Ginsenoside R_e on NOS activity in spermatozoa

Human spermatozoa exhibited a detectable NOS activity in both fertile and infertile samples, measured by the ^{3}H-arginine to ^{3}H-citrulline conversion assay (Fig. 2). Results from Western blot analysis showed the expressions of both ecNOS and iNOS proteins in fertile and infertile spermatozoa as bands at 140 kDa and 130 kDa, respectively (Fig. 3). The NOS activity was lower in infertile spermatozoa than that in fertile ones (Fig. 2, 3). Ginsenoside R_e significantly enhanced both fertile and infertile sperm NOS activity in a concentration-dependent manner. Also, L-NAME completely abolished the effects of Ginsenoside R_e (Fig. 2 and 3).

Effect of Ginsenoside R_e on NO production in spermatozoa

NO production (shown by the accumulation of nitrite) in fertile sperm was significantly higher than that in asthenozoospermic infertile sperm. The levels of NO in both fertile and infertile spermatozoa treated with Ginsenoside R_e were significantly increased with the concentrations of Ginsenoside R_e as compared with control samples (Fig. 4). And again, L-NAME or LNAC completely blocked nitrite accumulation induced by Ginsenoside R_e, respectively (Fig. 4).

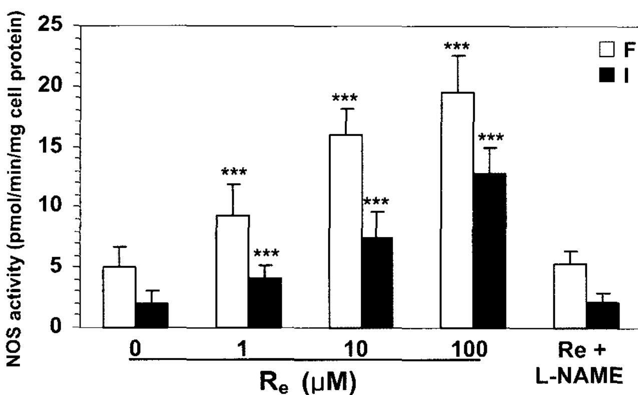

Fig. 2. Effects of different concentrations of Ginsenoside R_e, or L-NAME (NOS inhibitor) on fertile (□ F) and asthenozoospermic infertile (■ I) human sperm NOS activity at 2 h. Data represent mean ± SEM, n = 10, ***p < .001 vs. control.

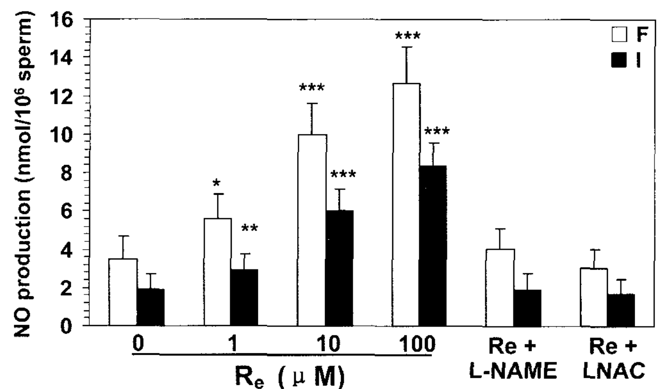

Fig. 4. Effects of different concentrations of Ginsenoside R_e, or L-NAME (NOS inhibitor) and L-NAME (NO scavenger) on fertile (□ F) and asthenozoospermic infertile (■ I) human sperm NO production (shown by the accumulation of nitrite) at 2 h. Data represent mean ± SEM, n = 10, *p < .05; **p < .01, ***p < .001 vs. control.

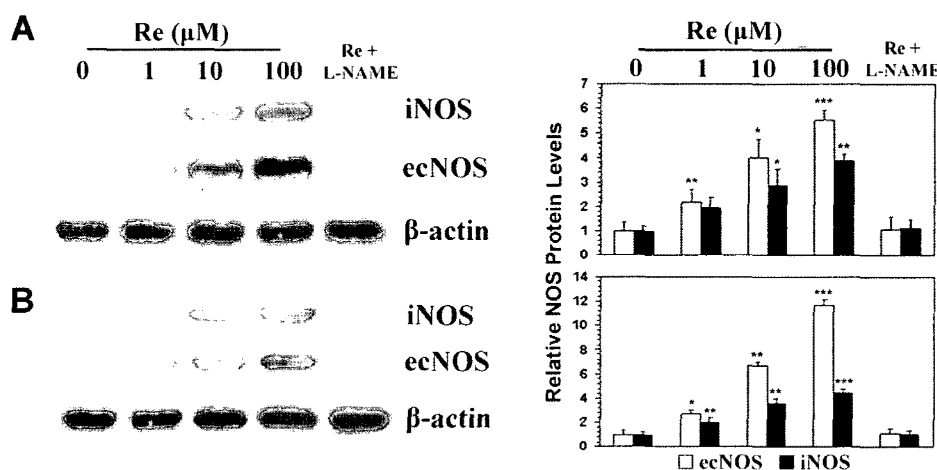

Fig. 3. Western blot analysis showing the presence of both ecNOS and iNOS proteins in fertile (A) and infertile (B) spermatozoa (left). Ginsenoside R_e significantly enhanced both fertile and infertile sperm NOS activity in a concentration-dependent manner, and L-NAME completely abolished the effects of Ginsenoside R_e. Relative amounts of NOS proteins were shown at the right. Values are mean ± SEM, n = 10, *p < .05; **p < .01, ***p < .001 vs. control.

DISCUSSION

It is known that the capacity of human sperm fertilization is principally dependent on sperm motility and sperm membrane integrity; fertilization will be impaired if they are damaged. Human spermatozoa, especially abnormal spermatozoa in motion, morphology, and functions, can generate reactive oxygen species (ROS), such as superoxide anion (O_2^-) and hydrogen peroxide (Alvarez *et al.*, 1987; Aitken and Clarkson, 1987; Iwasaki and Gagnon, 1992), and are especially sensitive to oxidative damage because of their high concentration of polyunsaturated fatty acids and their relatively low levels of antioxidant enzymes (Alvarez *et al.*, 1987; Aitken and Clarkson, 1987; Iwasaki and Gagnon, 1992; Jone *et al.*, 1978). The effects of ROS to sperm plasma membrane are thought to be responsible for loss of sperm motility and other sperm functions (Alvarez *et al.*, 1987; Aitken and Clarkson, 1987; Iwasaki and Gagnon, 1992; Jone *et al.*, 1978). And, sperm motility can be improved by exogenous antioxidants/ ROS scavengers (Rees *et al.*, 1990; Zheng and Zhang, 1997; Suzuki *et al.*, 2003; Keskes-Ammar *et al.*, 2003). NO, despite being a free radical itself, can protect cells from peroxidative damages as a free radical scavenger by inactivating and inhibiting O_2^- (McCall *et al.*, 1989; Clancy *et al.*, 1992). Furthermore, recent studies have demonstrated that NO can be generated by spermatozoa themselves and both endogenous and exogenous NO play an important role in improvement of sperm functions (Lewis *et al.*, 1996; Revelli *et al.*, 1999; Hellstrom *et al.*, 1994; Zhang and Zheng, 1996). In the present study, NO (shown by the accumulation of nitrite, the stable end–product of the NOS/NO pathway) was also detected in both fertile and asthenozoospermic infertile spermatozoa, and NO production in fertile sperm was significantly higher than that in asthenozoospermic infertile sperm. Presence of NOS activity and both ecNOS and iNOS proteins in the spermatozoa, and their different levels in fertile and asthenozoospermic infertile individuals supported a physiological role of NO in maintenance of sperm motility. Importantly, the present study has provided significant evidences that Ginsenoside R_e, which stimulated NO production in many cells and tissues (Li *et al.*, 2000; Scott *et al.*, 2001; Kim *et al.*, 2003; Bai *et al.*, 2004) by inducing NOS activity, markedly increased sperm motility and sperm NO production in both fertile and asthenozoospermic infertile individuals in a concentration-dependent manner, especially asthenozoospermic samples. Simultaneously, sperm NOS activity and NOS protein levels have also been enhanced after spermatozoa were incubated with Ginsenoside R_e. This further confirmed that Ginsenoside R_e improved human sperm motility by induction of NOS to increase sperm NO production, and free radical scavenging function of NO may be involved this mechanism.

Exogenous NO donors have indeed been shown to enhance sperm motility, viability, hyperactivation, capacitation and sperm binding to the zona pellucida (Hellstrom *et al.*, 1994; Zhang and Zheng, 1996; Zini *et al.*, 1995; Herrero *et al.*, 1999; Yeoman *et al.*, 1998; Sengoku *et al.*, 1998). However, majority of this NO donors are toxic on spermatozoa at high concentrations (Zhang and Zheng, 1996; Zini *et al.*, 1995; Tomlinson *et al.*, 1992; Weinberg *et al.*, 1995). The effects of NO on sperm functions are biphasic: at lower concentrations, NO improves sperm functions (Hellstrom *et al.*, 1994; Zhang and Zheng, 1996; Zini *et al.*, 1995; Herrero *et al.*, 1999; Yeoman *et al.*, 1998; Sengoku *et al.*, 1998); high concentration NO has a deleterious effect on sperm motility (Zhang and Zheng, 1996; Zini *et al.*, 1995; Tomlinson *et al.*, 1992; Weinberg *et al.*, 1995). Moreover, NO has a very short half life and its concentration in the samples is difficult to be controlled. Thus, it is a more physiological and more secure way to induce sperm endogenous NOS activity to generate NO using some drugs which possess competences of inducing cellular NOS activity, especially the effective components of medicinal herbs.

In addition to inducing sperm endogenous NOS activity, the mechanism by which Ginsenoside R_e exerts its role of increasing human sperm motility is involved partially its property as a free radical scavenger (Chen, 1996; Chu and Chen, 1990). Some effective components of medicinal herbs which possess antioxidative/free radical scavenging properties have been reported to improve sperm functions *in vitro* and *in vivo* (Rees *et al.*, 1990; Zheng and Zhang, 1997; Suzuki *et al.*, 2003).

In summary, our results suggest that Ginsenoside R_e is beneficial to sperm motility, and we revealed for the first time that Ginsenoside R_e improved human sperm motility by inducing sperm NOS activity to increase endogenous NO production. It is possible that Ginsenosides are used for treatment of asthenozoospermic infertility, or for fertilization *in vitro* as a sperm motility stimulant.

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