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Seminal superoxide dismutase activity and its relationship with semen quality and *SOD* gene polymorphism

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Abstract
Purpose Superoxide dismutase (SOD) is an important component of antioxidative defense systems and plays an important role in protecting spermatozoa from oxidative damage. In this study, we assessed seminal SOD activity, its association with semen parameters, and also genetic and non-genetic factors contributing to the determination of SOD activity in infertile men.
Methods Semen samples were obtained from 435 male infertility patients. Sperm DNA damage levels were detected with the Tdt-mediated dUTP nick end labelling (TUNEL) assay. Four single nucleotide polymorphisms (SNPs) in *SOD2* and *SOD3* genes were genotyped using OpenArray platform.
Results We found that seminal SOD activity was positively associated with sperm concentration and overall motility, whereas inversely with sperm DNA fragmentation. In addition, infertile men with *SOD2* rs4880 CC variants showed a low level of SOD activity when compared with TT carriers (Mean ± SD: 268.3±102.3 and 342.8±98.2, respectively, P=0.005). Those who consumed vitamin C/E (≥3 times per week) had a significantly higher SOD activity level than those who did not (mean ± SD: 379.8±93.3 and 332.2±94.9, respectively, P=0.001).
Conclusions Seminal SOD activity and other factors influencing SOD activity play a role in determining sperm fertilization potential and male infertility.

Keyword SOD activity · Semen quality · Polymorphism · Vitamin C · Vitamin E · Male infertility

Introduction

Oxidative stress has been implicated in the pathophysiology of damage to human spermatozoa [1, 2]. It is a consequence of an imbalance between Reactive Oxygen Species (ROS) production and degradation. Germ cells produce physiological amounts of ROS that are required for maturation, capacitation, the acrosome reaction, and oocyte fusion under normal physiological conditions [3]. However, excessive generation of ROS can impair normal sperm function by attacking the fluidity of the sperm plasma membrane and the integrity of sperm DNA, and ROS-induced DNA damage could accelerate the process of germ cell apoptosis, resulting in the decline in sperm counts associated with male infertility [2].

To protect spermatozoa from oxidative damage, seminal plasma has a highly specialized ROS scavenger system, containing superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT). Among them, SOD is known to be the most important antioxidant enzyme. Leydig and Sertoli cells have been reported to produce SOD in the testis [4, 5]. A significantly lower semen SOD activity was found in oligoasthenozoospermic cases, when compared to normospermic men [6]. Previous studies have detected a positive correlation between seminal SOD with abnormal sperm morphology, percentage of dead sperms, sperm

Capsule SOD activity was found to be associated with semen quality and in individuals consuming vitamins C and E, a significantly higher expression level of SOD was observed.

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concentration and overall motility in male infertility patients [7, 6]. However, other researchers reported a nonsignificant correlation between SOD activity in seminal plasma and semen quality [8].

Thus, the purpose of this work was to evaluate SOD activity in seminal plasma of male infertility cases in a Chinese population, and its relationship with selected human semen parameters and sperm DNA fragmentation. As is known to all, genetic factors and life style factors might contribute to the determination of SOD activity. This study includes also these influence factors of SOD activity. Therefore, knowledge of activity of SOD in seminal plasma, its association with sperm parameters and influencing factors could be a useful tool for determining sperm fertilization potential and could improve the diagnosis and the prevention of male infertility.

Materials and methods

Subjects and sample collection

The study was approved by the Ethics Review Board of Nanjing Medical University. All the studies involving human subjects were conducted in full compliance with government policies and the Declaration of Helsinki. A total of 709 infertile patients diagnosed with unexplained male factor infertility were drawn from the Center of Clinical Reproductive Medicine between April 2005 and March 2009 (NJMU Infertility Study). All patients underwent at least two semen analyses, a series of physical examinations and serum determination, which helped us to exclude 274 individuals: 210 azoospermia (6 obstructive azoospermia), 7 with cryptorchidism,11 with abnormal karyotype, 12 with Y-chromosome microdeletions and 34 secondary sterility cases . In the final analysis, 435 idiopathic infertility patients aged 24 to 42 years were included. All participants were ethnic Han Chinese and completed an informed consent and a questionnaire including detailed information, such as age, cigarette smoking, alcohol intake, tea and vitamin C/E consumption, and abstinence time. Each subject donated 5 ml of blood for genomic DNA extraction. In addition, the semen samples were were obtained in private by masturbation into a sterile widemouth and metal-free glass container after a recommended at least 3-day sexual abstinence. The semen samples, frozen at −70 °C, were used for routine semen analysis, CASA motion analysis, the assessment of sperm DNA fragmentation, and the SOD activity.

Semen quality analysis

After liquefaction at 37 °C for 30 min, conventional semen analysis was conducted according to WHO criteria [9] by using Micro-cell slide and the computer-aided semen analysis (CASA, WLJY 9000, Weili New Century Science and Tech Dev.). Recorded semen parameters were sperm concentration, sperm motility, and CASA motion parameters, including straight-line velocity (VSL), curvilinear velocity (VCL), and linearity (LIN).

Determination of seminal SOD activity

SOD activity in seminal plasma was measured by the inhibition of nitrite (NIT) reduction due to the superoxide anion generated by the combination xanthine and xanthine oxidase. SOD assay kit (NO.A001-1, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) was used to assess the SOD activity. One unit of SOD was the amount that caused a 50 % inhibition in the rate of NIT reduction. The SOD activity was expressed as U/mL seminal plasma.

DNA fragmentation analysis

As described previously [10], the Terminal-deoxynucleoitidyl Transferase Mediated Nick End Labeling (TUNEL) assay was performed to detect DNA fragmentation. We used the APO-DIRECT kit (BD Biosciences Pharmingen, SanDiego, CA, USA) according to the manufacturer's protocol. Briefly, semen samples were thawed in a 37 °C water bath and immediately diluted with buffer (0.15 mol/l NaCl, 0.01 mol/l Tris, 0.001 mol/l EDTA, pH 7.4) to obtain a sperm concentration of $1–2\times10^6$/ml. Washed sperm were resuspended in 2 % paraformaldehyde for 30 min at room temperature. After being rinsed with PBS, samples were resuspended in permeabilisation solution (0.2 % Triton X-100, 0.1 % sodium citrate) for 10 min on ice. Fifty millilitres of TUNEL reagent were then added to the sample. For each batch, samples that were not treated with the Tdt enzyme were used as negative controls, and samples treated with DNase I were included as positive controls. After incubation for 1 h at 37 °C, samples were analysed immediately by flow cytometry (FACSCalibur; BD Biosciences Pharmingen).

Genotyping

Given that the *SOD2* and *SOD3* genes are all active in the ROS detoxification pathway, we selected four potential functional polymorphisms in *SOD2* and *SOD3* for genotyping. All of these single nucleotide polymorphisms (SNPs) have a reported minor allele frequency of ≥0.05 in the general Han Chinese population.

Briefly, DNA was extracted from leukocyte pellets of the venous blood by phenol-chloroform extraction with proteinase K digestion, and frozen until use. Genotype analysis was done using the OpenArray platform (Applied Biosystems, Foster City, CA, USA), which employs a chip-based TaqMan genotyping technology. Genotyping was conducted

according to the manufacturer's standard protocols, and genotype calls were made by OpenArray SNP Genotyping Analysis Software version 1.0.3. For quality control, 10 % of the samples were randomly genotyped twice by different individuals, and the reproducibility was 100 %. To confirm the genotyping results, selected PCR-amplified DNA samples (n=2, for each genotype) were examined by DNA sequencing and the results were also consistent.

Statistical analyses

All the statistical analyses were performed using the STATA 11.0 (STATA Corp, College Station, Texas). Sperm DNA fragmentation and sperm concentration were normalized by natural logarithm (ln) transformation. Linear regression models were used to estimate the correlations of seminal SOD activity with ln-transformed sperm fragmentation, ln-transformed sperm concentration values and other semen parameters after adjusted for age, smoking, drinking status, tea consumption and vitamin C/E using. The effects of selected individual characteristics on seminal SOD activity were analyzed by oneway ANOVA. Multivariate logistic regression analysis was employed to examine the effects of *SOD* polymorphisms on seminal SOD activity after adjusted for age, smoking, drinking status, tea consumption and vitamin using. The data were expressed as the mean ± SD. All P-values were two-sided and the significance level was set at $P<0.05$.

Results

Characteristics of the study population

Subjects' demographic information and semen quality in 435 infertile men were presented in Table 1. All these participants were Han Chinese with an average age of 28.7 years, mean Body Mass Index (BMI) of 23.6 kg/m^2, an average abstinence period of 5.8 days. One hundred and ninety-four (44.6 %) participants were never smoker, 207 (47.6 %) were current smoker, and 34 (7.81 %) were former smoker. Of the 435 semen samples evaluated, the mean sperm concentration (10^6/ml) and sperm motility (% motile) were 69.6 and 57.9, respectively. Eighty-one (18.6 %) participants having sperm concentration values less than 20 million sperm/ml and 220 (50.6 %) having sperm motility values less than 50 % motile sperm. In addition, the sperm DNA damage was also detected, and the average sperm DNA fragmentation was 18.7 %, with 161 (37.0 %) having sperm DNA damage higher than 20 % TUNEL.

Table 1 Subjects' demographic information and semen quality in 435 infertility men

Characteristic	Mean ± SD	No (%)
Age (years)	28.7±3.35	
Smoking status		
Never smoker		194 (44.6)
Current smoker		207 (47.6)
Former smoker		34 (7.81)
Body mass index (kg/m^2)	23.6±3.23	
Abstinence time (days)	5.8±4.47	
3		62 (14.2)
4–5		205 (47.1)
6–7		117 (26.9)
≥8		51 (11.7)
Semen parameters		
Sperm concentration (10^6/ml)	69.6±59.4	
Subjects <20 million sperm/ml[a]		81 (18.6)
Sperm motility (% motile)	57.9±25.9	
Subjects <50 % motile sperm[a]		220 (50.6)
Sperm DNA damage (%)	18.7±15.6	
Subjects >20 % TUNEL		161 (37.0)

[a] According to WHO (1999)

Associations between individual characteristics and seminal SOD activity in infertile subjects

The individual characteristics and the effects on seminal SOD activity levels in 435 infertile men were shown in Table 2. We found that individuals who consumed vitamin C/E (≥3 times per week) had significantly higher SOD activity levels than those who did not (mean ± SD: 379.8±93.3 versus 332.2± 94.9, respectively, P=0.001). However, other characteristics, such as age, alcohol intake, smoking status, and tea consumption appeared to have no obvious effects on seminal SOD activity.

The correlation between seminal SOD activity and semen quality

As shown in Table 3, there was a positive association between sperm concentration and seminal SOD activity. The ln-transformed sperm concentration (10^6/ml) increased, on average, by 0.002 (95 % confidence interval (CI): 0.001, 0.003, $P<0.001$) per 1-unit increase in the seminal SOD activity. Seminal SOD activity was also significantly associated with the percentage of sperm motility (coefficient: 0.039, 95 % CI: 0.010, 0.068, P=0.008). Additionally, a negative relationship was found between ln-transformed sperm DNA fragmentation (%) and seminal SOD activity (coefficient: −0.001, 95 % CI: −0.002, −0.0003, P=0.014). However, no associations were

Table 2 Distribution of SOD activity results, exposure variables, and potential confounders in 435 infertility men

Variables	Subjects *N* (%)	SOD activity (Mean ± S.D. U/ml)	*P*[a]
Age (years)			
<30	205 (47.1)	339.8±102.5	0.432
≥30	230 (52.9)	332.5±91.3	
Smoking status			
Never	194 (44.6)	332.1±104.4	0.2537
Current	207 (47.6)	335.7±90.8	
Former	34 (7.81)	361.9±86.7	
Drinking status			
Yes	65 (14.9)	323.5±94.5	0.2805
No	370 (85.1)	338.2±97.3	
Tea consumption			
Yes	130 (29.9)	343.6±86.78	0.311
No	305 (70.1)	333.1±100.8	
Vitamin C/E using (≥3 times per week)			
Yes	59 (13.6)	379.8±93.3	**0.0013**
No	376 (86.4)	332.2±94.9	

[a] Oneway ANOVA

Data in *boldface* represent $P<0.05$

found between the seminal SOD activity and CASA motion parameters.

Association between *SOD2*, *SOD3* polymorphisms and seminal SOD activity in infertile subjects

The associations between the four defined functional polymorphisms and seminal SOD activity were evaluated by multiple linear regressions in Table 4. We found that individuals with *SOD2* rs4880 CC variants showed a low level of SOD activity when compared with common TT genotype carriers (Mean ± SD: 268.3±102.3 and 342.8±98.2, for CC and TT, respectively, $P=0.005$). However, we did not find any significantly association between other studied SNPs (*SOD2* rs5746136, *SOD3* rs2536512, and *SOD3* rs2695232) and seminal SOD activity in our population.

Table 3 Correlations of seminal SOD activity with semen parameters and sperm DNA fragmentation after adjusted for age, smoking, drinking status, tea consumption and vitamin using in 435 infertility men

Variables	Coef.[a]	Std. Err	95 % Conf. Interval	*P*-values
Semen quality parameters				
Sperm concentration (10^6/ml) [b]	0.002	0.0005	0.001, 0.003	**<0.001**
Sperm motility (%)	0.039	0.0147	0.010, 0.068	**0.008**
CASA motion parameters				
VSL (μm/s)	0.840	0.478	−0.100, 1.78	0.080
VCL (μm/s)	0.313	0.372	−0.418, 1.04	0.401
LIN (%)	0.913	0.465	−0.043, 1.87	0.092
Sperm DNA fragmentation (%)[b]	−0.001	0.0005	−0.002, −0.0003	**0.014**

[a] Adjusted linear regression coefficients

[b] ln-transformed

Data in *boldface* represent $P<0.05$

Discussion

In the present study, we evaluated SOD activity in seminal plasma of 435 male infertility patients, and their relationship with selected human semen parameters and sperm DNA fragmentation, and investigated genetic factors that might contribute to the determination of SOD activity as well. Our analysis showed that SOD activity in seminal plasma was positively associated with sperm concentration and overall motility, whereas inversely with sperm DNA fragmentation. In addition, we found that *SOD2* rs4880 variant genotype was associated with a low level of SOD activity, and those who consumed vitamin had significantly higher SOD activity levels comparing to those who did not.

ROS affect sperm membrane fluidity by oxidising the polyunsaturated fatty acids (PUFAs), and sperm motility is

Table 4 Effects of SOD polymorphisms on seminal SOD activity in 435 infertility men

Genotype	Subjects *N* (%)	SOD activilty		*P*[a]
		Mean ± S.D.(U/ml)	Range (U/ml)	
SOD2 rs4880				
TT	292 (67.1)	342.8±98.2	83.5–740.2	Reference
TC	124 (28.5)	329.6±88.7	101.2–621.3	0.437
CC	19 (4.4)	268.3±102.3	79.8–421.5	**0.005**
SOD2 rs5746136				
GG	116 (26.7)	344.5±97.3	108.1–740.2	Reference
GA	214 (49.2)	332.6±99.3	79.8–621.3	0.572
AA	105 (24.1)	332.7±91.4	83.5–556.0	0.666
SOD3 rs2536512				
GG	344 (79.1)	337.0±96.9	79.8–740.2	Reference
GA	83 (19.1)	332.9±98.3	83.5–522.0	0.944
AA	8 (1.8)	315.0±85.4	211.4–434.8	0.818
SOD3 rs2695232				
CC	169 (38.9)	348.6±86.4	86.3–556.0	Reference
CT	183 (42.1)	326.0±100.1	79.8–621.3	0.091
TT	83 (19.1)	331.5±107.2	108.1–740.2	0.419

[a] Adjusted for age, smoking, drinking status, tea consumption and vitamin using

Data in *boldface* represent $P<0.05$

closely correlated with sperm membrane integrity. As SOD is an important element of ROS scavenger system that protects sperm from oxidative damage, the positive relationship between seminal SOD activity and sperm motility found in this study seems biologically reasonable. This result also confirms to the previous observations of Murawski et al. [6]. Additionally, a positive correlation between seminal SOD activity and sperm concentration, and a negative relationship between sperm DNA fragmentation and seminal SOD activity were also found in this study, suggesting a critical role of SOD in protecting sperm form ROS-induced DNA damage which could accelerate the process of germ cell apoptosis, resulting in the decline in sperm counts associated with male infertility [2].

Of the four defined functional polymorphisms in *SOD2* and *SOD3* studied here, we found a strong association between *SOD2* rs4880 (nsSNP/Val16Ala) and lower SOD activity (Mean ± SD: 268.3±102.3 and 342.8±98.2, for CC and TT, respectively, P=0.005). SOD2 (MnSOD) plays a critical role in protecting cells from free radicals and oxidative damage, and localization of SOD2 into the mitochondrial matrix is essential for it to protect sperm from oxidation [11]. It has been demonstrated that the *SOD2* Ala variant at a mitochondrial targeting sequence allows for efficient MnSOD import into the mitochondrial matrix, while the Val variant causes partial arrest of the precursor within the inner membrane and decreased formation of the active MnSOD tetramer in the mitochondrial matrix [12]. Since the mitochondria are protected from H_2O_2 by MnSOD enzyme, cells could become susceptible to H_2O_2-related damages when the activity of MnSOD in the mitochondria is reduced. Thus, we could speculate that lower SOD activity in seminal plasma caused by the Val16Ala polymorphism may result in male infertility. To test the hypothesis, our previous study examined this SNP with male infertility risk in a case–control study, and found that *SOD2* Val16Ala (rs4880) variant genotypes were associated with a significantly higher risk of male infertility [13].

With respect to vitamin supplementation, we observed individuals who consumed vitamin C/E (≥3 times per week) had significantly higher SOD activity levels than those who did not (mean ± SD: 379.8±93.3 versus 332.2±94.9, respectively, P=0.001). The role of ascorbic acid (vitamin C) and tocopherol (vitamin E) in the prevention of the damage caused by free radicals and lipid peroxidation was well established [14]. Vitamin E, a lipid-soluble antioxidant, is able to repair oxidizing radicals directly, preventing the chain propagation step during lipid peroxidation, thus playing a vital role in protecting cell membranes [15]. Vitamin C is a water-soluble antioxidant that exerts its antioxidant effect through scavenging ROS by very rapid aqueous phase electron transfer, and preventing initiation of lipid peroxidation [16]. Previously, a systematic review of randomized studies was conducted to evaluate the effects of oral antioxidants (vitamins C and E, zinc, selenium, folate, carnitine and carotenoids) on sperm quality and pregnancy rate in infertile men, and showed an improvement in either sperm quality or pregnancy rate after antioxidant therapy [17]. Thus, vitamin C/E using might have a unique effect on SOD activity and a preventive role in clinical condition caused by oxidative stress-derived male infertility.

It's worth mentioning that cigarette smoking, one of the main exogenous sources of oxidants, is correlated with alterations in sperm concentration, motility, morphology, and sperm function [18]. It has been long reported that ROS are found in high concentrations in semen of infertile smoking men [19]. However, in this study, we found no change of SOD activity in patient smokers. It might be possible that the use of vitamins combated the negative effect of smoking on SOD activity. Thus, further studies are needed to validate it.

In summary, SOD activity in seminal plasma was positively associated with sperm concentration and overall motility, whereas inversely with sperm DNA fragmentation. Additionally, *SOD2* rs4880 was associated with low levels of SOD activity, and those who consumed vitamin had significantly higher SOD activity levels, when compared to those who did not. Thus, knowledge of activity of SOD in seminal plasma, its relationship with sperm parameters and influencing factors could be a useful tool for determining sperm fertilization potential and could improve the diagnosis and the prevention of male infertility.

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Conflict of interest No competing financial interests exist.

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