Low NRF2 mRNA Expression in Spermatozoa from Men with Low Sperm Motility

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Antioxidant genes and enzymes play important roles in human spermatogenesis. Although low levels of antioxidant enzyme expression are associated with poor sperm quality, it is not clear whether mRNA expression of antioxidant genes is lower in these men than in normozoospermic men. In this study, 55 asthenozoospermic and oligoasthenozoospermic patients and 65 controls were recruited. Quantitative real-time reverse transcription PCR was performed and the abundance of mRNA of four antioxidant genes known to be important to spermatogenesis were evaluated. These genes were nuclear factor erythroid 2-related factor 2 (NRF2), catalase (CAT), glutathione S-transferase Mu 1 (GSTM1), and superoxide dismutase isoenzyme 2 (SOD2). Results showed the level of NRF2 mRNA expression to be significantly lower in patients than in controls ($P < 0.001$), but no statistically significant difference in the level of SOD2, CAT, or GSTM1 gene expression was observed between the two groups. A significant correlation was observed between the level of NRF2 mRNA expression and specific sperm function parameters, including concentration, progressive motility, immotility, vitality, and morphology (all $P < 0.01$). NRF2 expression was also found to be associated with seminal SOD activity and mRNA levels of the CAT and SOD2 genes (all $P < 0.05$). Therefore, our data demonstrated that the level of NRF2 mRNA expression is significantly lower in human males with low sperm motility and correlated with specific sperm function parameters. This suggests that NRF2 is important to spermatogenesis and may serve as a useful biomarker in the prediction of male infertility.

Keywords: biomarker; mRNA expression; nuclear factor erythroid 2-related factor 2; spermatogenesis; sperm motility

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Hemachand et al. (2002), where they are thought to take part in detoxification and protective processes. Seminal catalase catalyzes the degradation of H₂O₂ to oxygen and water, and it is involved in modulation of the levels of ROS to maintain normal sperm function and protect spermatozoa from potential toxic ROS (Jeulin et al. 1989).

Many antioxidant enzymes share a common regulatory factor, nuclear factor erythroid 2-related factor 2 (NRF2). In response to oxidant injury, NRF2 was found to activate the transcription of antioxidant enzymes by binding directly to antioxidant response elements (AREs) in their promoters (Kensler et al. 2007). The importance of NRF2 in antioxidant defense has been proven in numerous studies (Chan and Kan 1999; Kensler et al. 2007; Nakamura et al. 2010). NRF2 gene deficiency reduced constitutive and inducible expression of many antioxidant genes, including SODs, CAT, and GSTs (Chan and Kan 1999), and NRF2 knockout mice were less fertile than their wild-type and heterozygous littermates (Nakamura et al. 2010). For this reason, NRF2 is also considered to be important to spermatogenesis.

Previous studies have shown that seminal SOD and catalase enzyme activities are positively correlated with sperm quality (Aitken et al. 1996; Siciliano et al. 2001; Shiva et al. 2011) and genetic variations in the Nrf2 promoter that involve significant decreases in transcriptional capability are associated with oligoasthenozoospermia in humans (Yu et al. 2012). However, little is known about the relationship between antioxidant gene expression and sperm function. Recent progress has demonstrated that the mRNA profiles of ejaculated spermatozoa may be an effective substitute for transcripts in male reproductive system, and they may be suitable for use as clinic biomarkers (Ostermeier et al. 2002; Miller and Ostermeier 2006). In this way, analysis of mRNA expression in ejaculated spermatozoa may be a good way to study the roles of ROS and antioxidant defense in spermatogenesis. Unlike other techniques, this method would not require biopsies of the testes or epididymis.

The purpose of this study was to determine whether the level of mRNA expression of antioxidant genes in spermatozoa is associated with sperm function. The levels of mRNA expression of the antioxidant genes NRF2, GSTM1, SOD2 and CAT in ejaculated spermatozoa and the seminal SOD activity were measured and compared between men with low and high sperm motility. The results obtained may facilitate understanding of the molecular basis of defective sperm function in humans and may provide potential biomarkers of male infertility.

Methods

Study subjects

This study was approved by the institutional review boards of Guangzhou Medical University, and was conducted according to the guidelines laid down in the Declaration of Helsinki. Study subjects were ethnic Han Chinese men from Guangzhou City and surrounding regions. Asthenozoospermia or oligoasthenozoospermia patients who visited the Reproductive Medicine Center in the Third Affiliated Hospital of Guangzhou Medical University for semen analysis were recruited from November 2011 to June 2012 under the following inclusion criteria: 1) age between 25 to 50 years; 2) consistent results in at least two semen quality examinations; and 3) sperm progressive motility below 32%, which is the lower reference limit of progressive motility as established the 5th edition of World Health Organization (WHO) laboratory manual for the examination and processing of human semen (2010). Normozoospermia controls matched for age and smoking status were recruited from men who visited Reproductive Medicine Center for fertility consultation. In total, 120 males (55 patients and 65 controls) successfully underwent seminal SOD activity assays and qRT-PCR analysis of their spermatozoa samples.

Sample collection

The laboratory evaluation of patients included at least two semen analyses per subject. Semen was obtained by masturbation after 2 to 5 days of abstinence. Samples were collected into sterile containers, allowed to liquefy at 37°C for 30 minutes, and analyzed for sperm morphology, concentration and motility by computer-assisted semen analysis (CASA) according to the criteria of the 5th edition of WHO laboratory manual for the examination and processing of human semen (2010). Aliquots of semen samples were snap-frozen in liquid nitrogen and stored at −80°C until analysis.

Superoxide dismutase activity assay

Total SOD activity was assayed using an SOD assay kit and a commercial SOD standard (Sigma, St. Louis, MO, U.S.) according to the manufacturer’s instructions. The protein concentration of each sample was determined using BCA assay (Pierce, U.S.). Total SOD activity of each sample was converted into units per mg of total protein in seminal plasma (U/mg).

RNA extraction and first strand cDNA synthesis

Total RNA was isolated from the sperm pellet of each subject (totally 20 × 10⁸ spermatozoa) using RNeasy Plus Universal Mini Kit (Qiagene, Shanghai, China) according to its protocol. The RNA concentration was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., FL, USA) and stored at -80°C until preparation of complementary DNA (cDNA).

Quantitative real-time reverse transcription PCR

All RNA was reverse transcribed into cDNA (Takara, Dalian, China), and following qPCR was performed using SYBR Green Super-mix and iCycler (Bio-Rad, Hercules CA, USA). cDNA about (50 ng per sample) was used for amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), NFR2, SOD2, GSTM1, and CAT. The thermal cycling program included an initial incubation at 95°C for 2 min, followed by 49 cycles of 95°C for 5 s and 60°C for 20 s. A final 65°C to 95°C step was used to form the melt curve. According to literature (Wan and Diaz-Sanchez 2006; Garbin et al. 2009; Corrales et al. 2011), pre-designed primers of GAPDH (F: 5'-TGGTGAGGGTGAGTTGACG-3' and R: 5'-CCATGTAGTTAGGTCAATGAA-3'), NFR2 (F: 5'-TTCAGCCAGCCACACATC-3' and R: 5'-CTGAGCCGAAGAAACCTCATTGTC-3'), CAT (F: 5'-GTTACTCAGGGTGCCGATTTCACT-3' and R: 5'-GAA GTTCCTGACCGTTTTCTTCTG-3'), GSTM1 (F: 5'-CAGTGCACGCATCTTCTTT-3' and R: 5'-CGCCCAATACGGCAAAATC-3'),
and SOD2 (F: 5′-GTGGAGACCACAGGGGAGTT-3′ and R: 5′-GTGGAATAAGGCCTGTTGTTCCTT-3′) were employed in the experiments (Generay, Shanghai, China). Values recorded for quantification were the fractional cycle numbers (Ct) where the background corrected amplification curves crossed a threshold value. The threshold value was set within the log-linear phase of the amplification curves. Two replicates of each reaction were performed and the CT values were averaged. The 2^ΔΔCT was calculated to represent the levels of antioxidant genes after normalization to that of GAPDH, where ΔCT = (CT, antioxidant genes-CT, GAPDH).

**Statistical analysis**

Levels of antioxidant enzyme activities (U/mg protein for SOD) and the enzyme mRNA expression (2^ΔΔCT) are expressed as mean ± standard derivations. All data were analyzed using the GraphPad Prism 4.0 for Windows (GraphPad Software, CA, U.S.) and the PASW Statistics 18 (SPSS Inc. U.S.). Association analysis was performed using the Spearman correlation test. Two-sided P values of 0.05 were considered statistically significant.

**Results**

**Semen parameters of study subjects**

The mean age, smoking status, and semen parameters of 65 controls and 55 patients are given in Table 1. These data showed that there were no significant differences in mean age or smoking status between the two study groups. However, basic semen parameters, including sperm concentration (44.8×10^6/mL vs 93.9×10^6/mL), rapid progressive motility (10% vs. 42%), total progressive motility (21% vs. 63%), and vitality (70% vs. 88%) were significantly lower in patients than in controls, whereas sperm immotility (73% vs. 30%) and abnormal morphology (97% vs. 92%) were significantly higher in patients than in controls (all P<0.01, Table 1).

**mRNA expression levels of antioxidant genes in spermatozoa**

Relative quantitative RT-PCR analysis was performed to determine the level of antioxidant gene mRNA expression in spermatozoa from men that differed in sperm motility. Gel electrophoresis of the PCR products and graphs of melt curve and melt peak indicated that targeted genes could be specifically amplified using designed primers and qRT-PCR (Figs. 1 and 2).

The level of NRF2 mRNA expression was significantly lower in patients than in controls (p = 0.0008, Table 2), and its abundance was about 43% that of controls (Fig. 3). The mRNA expression of the antioxidant enzymes CAT and SOD2 were lower in patients than in controls, but the differences were not statistically significant (P = 0.2035 and P = 0.1810 respectively, Table 2). GSTM1 expression levels were similar in patients and controls (P = 0.7181, Table 2).

**SOD activity in seminal plasma**

SOD activity in the patients and controls is presented in Fig. 4. The mean values of SOD activity in patients and controls were 2.25 ± 0.90 U/mg and 3.19 ± 1.02 U/mg respectively. These were significantly different (P < 0.001, Fig. 4).

**Correlations between NRF2 mRNA expression level and sperm quality, seminal SOD activity and other antioxidant gene expression levels**

Spearman correlation analysis showed NRF2 mRNA expression to be closely correlated with most sperm parameters, including concentration, progressive motility, vitality, immotility, and abnormal morphology (all P<0.01, Table 3). The level of NRF2 mRNA was also found to be closely associated with the expression of the mRNA of other antioxidant enzymes, such as CAT (P = 0.05, Table 3) and SOD2 (P<0.001, Table 3). It was also found to be associated with seminal SOD activity (P<0.05, Table 3).

**Discussion**

Oxidative stress related impairment of spermatogenesis is a common cause of male infertility (de Lamirande et al. 1997; Ochsendorf 1999). A biomarker capable of indi-
cating the antioxidant ability of the male reproductive system would be useful in both clinical diagnosis and determination of the role of ROS in spermatogenesis. Human spermatozoa contain large numbers of mRNAs, which originate from testis due to transcriptional inactivity of spermatozoa (Grunewald et al. 2005). The mRNAs detected in the testis and epididymis are consistent with those detected in sperm cells (Ostermeier et al. 2002; Miller and Ostermeier 2006). Therefore, mRNA in ejaculated spermatozoa may provide insight into the events of spermatogenesis (Ostermeier et al. 2002; Miller et al. 2005; Miller and Ostermeier 2006). The association between marker gene mRNA expression and sperm quality has been investigated in animals, and genes such as cystine rich secretary protein2 (CRISP2) and chaperone containing T complex subunit 8 (CCT8) have been identified (Arangasamy et al. 2011). Recent molecular analyses of mRNA populations in bull spermatozoa also support the conclusion that sperm RNA profiling is suitable for molecular diagnosis of male gamete quality (Gilbert et al. 2007).

In the current study, we demonstrated that NRF2 mRNA levels are associated with many sperm quality parameters, including concentration, motility, vitality, and the expression of both antioxidant genes and enzymes (Table 3). This suggests that NRF2 may be suitable for use as a biomarker in the prediction of ROS related male infertility. Several groups have found that low-quality human semen is associated with abnormal mRNA content for certain genes (Lambard et al. 2004; Steger et al. 2008; Liu et al. 2010). Lambard et al. (2004) found that eNOS and nNOS transcripts to be undetectable in highly motile sperms, but both were present in the low motile sperms. Weidner’s group also reported significantly aberrant protamine and Bcl2 mRNA expression in both testicular and

Fig. 1. Melt peak and melt curve of quantitative real-time RT-PCR performed using NRF2 gene primers.
Fig. 2. Gel electrophoresis of quantitative real-time RT-PCR products performed with NRF2 and GADPH primers. Lanes 1-3: subjects with low sperm motility. Lanes 4-7: subjects with high sperm motility. Lane 8: markers.

Table 2. Antioxidant gene expression in spermatozoa in the controls and patients.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Controls (n = 65)</th>
<th>Patients (n = 55)</th>
<th>P value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRF2(2^{-ΔCT})</td>
<td>2.16 ± 2.51</td>
<td>0.93 ± 0.94</td>
<td>0.0008*</td>
<td></td>
</tr>
<tr>
<td>CAT(2^{-ΔCT})</td>
<td>50.36 ± 32.68</td>
<td>41.00 ± 26.90</td>
<td>0.2035</td>
<td></td>
</tr>
<tr>
<td>GSTM1(2^{-ΔCT})</td>
<td>262.3 ± 134.2</td>
<td>277.6 ± 233.7</td>
<td>0.7181</td>
<td></td>
</tr>
<tr>
<td>SOD2(2^{-ΔCT})</td>
<td>3.79 ± 5.10</td>
<td>2.35 ± 3.16</td>
<td>0.1810</td>
<td></td>
</tr>
</tbody>
</table>

* Data are presented as mean value ± standard derivation.
* P value was calculated by independent sample t-test.*: statistically significant.
* 2^{-ΔCT} = 2^{(CT, antioxidant genes - CT, GAPDH)}.

Table 3. Correlations between NRF2 mRNA level and sperm quality, seminal SOD activity and antioxidant gene expression.

<table>
<thead>
<tr>
<th></th>
<th>Correlation coefficient</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm concentration</td>
<td>0.243</td>
<td>0.008*</td>
</tr>
<tr>
<td>Sperm progressive motility</td>
<td>0.311</td>
<td>0.006*</td>
</tr>
<tr>
<td>Sperm vitality</td>
<td>0.240</td>
<td>0.008*</td>
</tr>
<tr>
<td>Sperm immotility</td>
<td>−0.286</td>
<td>0.002*</td>
</tr>
<tr>
<td>Sperm abnormal morphology</td>
<td>−0.306</td>
<td>0.001*</td>
</tr>
<tr>
<td>Seminal SOD activity</td>
<td>0.232</td>
<td>0.048*</td>
</tr>
<tr>
<td>CAT mRNA level</td>
<td>0.226</td>
<td>0.050*</td>
</tr>
<tr>
<td>GSTM1 mRNA level</td>
<td>0.194</td>
<td>0.094</td>
</tr>
<tr>
<td>SOD2 mRNA level</td>
<td>0.815</td>
<td>0.000*</td>
</tr>
</tbody>
</table>

*: Correlation coefficient was calculated using the Spearman correlation test.
*: Correlation is significant at the 0.01 level (2-tailed).
ejaculated spermatozoa among infertile men (Steger et al. 2008). Differences in the mRNA level of the voltage-dependent anion channel (VDAC2) gene have been reported in men with low and high sperm motility (Liu et al. 2010). The present study shows NRF2 mRNA expression to be closely associated with sperm quality. In this way, NRF2 may be suitable for use as biomarker of ROS related defects in sperm function and determination of the etiology of male infertility.

Previous studies demonstrated that, in NRF2 knockout mice, spermatogenesis is highly susceptible to disruption by chronic oxidative stress (Nakamura et al. 2010) and that polymorphisms in NRF2 promoters can affect its mRNA expression and are associated with defective spermatogenesis in humans (Yu et al. 2012). Our data also suggested that the NRF2 gene plays a crucial role in human spermatogenesis, as men with low sperm motility were found to have significantly decreased NRF2 expression in the present study (Table 2, Fig. 3). We hypothesized that the decrease in NRF2 expression could have been caused by abnormal function of regulatory genes or by genetic variations in NRF2 itself. Because NRF2 is a key regulator of gene transcription during apoptosis, antioxidant defense, and cell survival (Kensler et al. 2007; Wakabayashi et al. 2010), its aberrant expression may affect not only ROS levels in the male reproductive system but also a broad range of genes involved in spermatogenesis. Therefore, aberrant NRF2 levels might be one of the molecular mechanisms underlying defective sperm function.

As in previous literature, we observed significantly lower activities of seminal SOD in the patients than in the controls (Fig. 4). SOD is one of the most important antioxidant enzymes in seminal plasma (Peeker et al. 1997), and it
has been reported a positive correlation between SOD activity in seminal plasma and semen quality parameters, such as sperm concentration, morphology and overall motility (Aitken et al. 1996; Siciliano et al. 2001; Shiva et al. 2011). Spermatozoa in semen are exceptionally well protected by SOD against superoxide radicals, which promotes spermatozoa survival and motility. Therefore, our data and those of others’ support the conclusion that a proper antioxidant enzyme level is important for ROS defense in spermatogenesis, especially for sperm maturation in the epididymis. These data are consistent with our observation that men with low SOD activity have low levels of NRF2 gene expression, as SOD enzymes are regulated by NRF2 (Chan and Kan 1999). Therefore, men with low sperm motility are likely to have decreased antioxidant capabilities at both the mRNA and enzyme levels.

In conclusion, the data collected in this study demonstrate that the mRNA level of the antioxidant gene NRF2 in spermatozoa and seminal SOD activity significantly decreased in men with low sperm motility. These findings suggest that an integrated antioxidant capability is required for normal human spermatogenesis and reduced antioxidant levels may be associated with defective sperm function. Because aberrant expression of NRF2 is associated with sperm function parameters, it might be used as biomarkers for ROS related male infertility. However, because this study was performed in a limited number of patients and controls, further investigation in a larger population is warranted. Experiments on the relationship between aberrant NRF2 mRNA level and low sperm motility should also be conducted to determine the mechanism underlying NRF2 and ROS related defects in sperm function.

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Conflict of Interest
All authors report no conflict of interest.

References


