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NRF2 mRNA Expression in Ejaculate Spermatozoa from Men with Asthenozoospermia and Oligoasthenozoospermia

Asthenozoospermi ve Oligoasthenozoospermi Erkek Olguların Ejekulat Spermatozoalarında NRF2 mRNA Ekspresyon Düzeyi

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ABSTRACT

Objectives: We aimed to determine whether the level of mRNA expression of antioxidant gene nuclear factor erythroid 2-related factor 2 (NRF2) in spermatozoa is associated with sperm function and also the seminal plasma superoxide dismutase (SOD) enzyme activity was to investigate in asthenozoospermia and oligoasthenozoospermia cases. **Material and Methods:** In this study asthenozoospermic and oligoasthenozoospermic 41 patients and normozoospermic 48 healthy individuals were included. Quantitative real-time reverse transcriptase polymerase chain reaction was used for detecting level of NRF2 mRNA expression in ejaculated spermatozoa and colorimetric method was also used to evaluate seminal plasma SOD activity. **Results:** Results revealed that both the level of NRF2 mRNA expression and the seminal plasma SOD activity levels have no statistically significant difference between the two study groups (respectively p=0.633, p=0.502). A significant correlation was not observed between the level of NRF2 mRNA expression and specific sperm function parameters (concentration, vitality, immotility, progressive motility, non-progressive motility, abnormal morphology) and also seminal plazma SOD activity (respectively; all p>0.05, p=0.553). Additionally there was no significant correlation between seminal plasma SOD activity and specific sperm function parameters (all p>0.05). **Conclusion:** These data indicated that NRF2 mRNA expression level and seminal plasma SOD activity did not show any significant difference in men with low sperm motility and that these factors were not related to specific sperm function parameters. As a result, it is thought that the NRF2 gene cannot be sufficient for using as a marker to determine the male infertility. In addition, this study showed that the SOD activity is not a sufficient marker to predict sperm fertilization potential.

Key Words: NFE2L2 protein, human; superoxide dismutase; spermatogenesis; sperm motility

ÖZET

Giriş: Asthenozoospermik ve oligoasthenozoospermik olguların spermatozoalarında antioksidan gen olan transkripsiyon faktörü nükleer faktör eritroid 2 ilişkili faktör 2 (NRF2)'nin mRNA ekspresyon düzeyinin sperm fonksiyonları ile olan ilişkisi ve ayrıca seminal plazma süperoksit dismutaz (SOD) enzim aktivitesinin araştırılması amaçlandı. **Gereç ve Yöntemler:** Bu çalışmaya, 41 asthenozoospermik ve oligoasthenozoospermik olgudan oluşan hasta grubu ve 48 sağlıklı bireyden oluşan normozoospermik kontrol grubu dahil edildi. Olguların ejekulat spermatozoalarında NRF2 mRNA ekspresyon düzeyini belirlemek için kantitatif real-time reverse transkriptaz polimeraz zincir reaksiyonu ve seminal plazma SOD aktivitesini değerlendirmek için ise kolorimetrik yöntem kullanıldı. **Bulgular:** Araştırmamız sonucunda, hasta grubunun hem NRF2 mRNA ekspresyon düzeyi hem de seminal plazma SOD aktivitesi kontrol grubu ile istatistiksel olarak karşılaştırıldığında anlamlı bir fark saptanmadı (sırasıyla; p=0.633, p=0.502). NRF2 mRNA ekspresyon düzeyinin spesifik sperm fonksiyon parametreleriyle (konsantrasyon, canlılık, hareketsiz sperm oranı, progresif hareketlilik, non-progresif hareketlilik, anormal spermatozoa oranı) ve ayrıca seminal plazma SOD aktivitesiyle aralarında anlamlı bir korelasyon saptanmadı (sırasıyla; tümü p>0.05, p=0.553). Seminal plazma SOD aktivitesinin de spesifik sperm fonksiyon parametreleri ile aralarında anlamlı bir korelasyon saptanmadığı belirlendi (tümü p>0.05). **Sonuç:** Bu veriler NRF2 mRNA ekspresyonunun ve seminal plazma SOD aktivitesinin düşük sperm hareketliliği olan erkeklerde anlamlı bir fark göstermediğini ve spesifik sperm fonksiyon parametreleriyle de ilişkili olmadığını göstermektedir. Bu veriler, NRF2 geninin erkek infertilitesini belirlemek için ve ayrıca SOD aktivitesinin sperm fertilizasyon potansiyelini tahmin etmek için bir belirteç olarak kullanılmasının yeterli olmadığını düşündürmektedir.

Anahtar Kelimeler: NFE2L2 protein, insan; süperoksid dismutaz; spermatogenez; spermin hareketliliği

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Infertility is defined as the non-occurrence of pregnancy in a year despite of a regular sexual intercourse of a pair of reproductive age without a contraception.[1] The human infertility affecting nearly 10% of couples of reproductive age around the World is a common medical problem that has become a public issue in recent years.[2]

Oxidative stress occurs when generation of reactive oxygen species (ROS) suppresses the antioxidant defenses.[3] The oxidative stress caused by ROS is one of the most important sources of low spermatogenesis.[4] The oxidative stress in spermatozoa is associated with male infertility because of defects in sperm motility and reduction in sperm vitality.[5] The harmful effects of ROS on sperm quality was shown in previous studies.[6] Although low concentration of ROS plays an important role in sperm physiology, high concentration of them may lead to some sperm pathologies such as lipid peroxidation and loss of motility and vitality.[5] It has been reported that the plasma membrane of spermatozoa contains high concentrations of polyunsaturated fatty acids. The spermatozoa cell membrane is highly sensitive to lipid peroxidation due to the characteristics of oxidation of unsaturated fatty acids in case of ROS.[7] Lipid peroxidation is generally associated with decreased sperm function and vitality.[5]

Spermatozoa and seminal plasma contain some important antioxidant enzymes, such as catalase (CAT), gluathione peroxidase (GPx) and superoxide dismutase (SOD), protecting most somatic cells against oxidative stress caused by ROS.[5,8] It is indicated that SOD enzymes play an important role to protect human spermatozoa against both oxidative stress and lipid peroxidation.[8,9]

One of the most important cellular defense mechanisms against oxidative stress is stimulated by the cap-n-collar basic leucine zipper (bZip) transcription factor nuclear factor-erythroid 2-related factor 2 (NRF2).[10] The main function of NRF2 is to activate the cellular antioxidant response by inducing the transcription of a wide array of genes that can combat the harmful effects of factors such as oxidative stress.[11]

Under basal conditions, NRF2-dependent transcription is represssed by the negative regulator protein Keap1, largely localized in the cytoplasm. When cells were exposed to agents like oxidative stress, NRF2 escapes Keap1-mediated repression and translocates to the nucleus. It is binds to ARE located in the promoters of many antioxidant enzyme genes in the nucleus, and activates the expression of ARE-dependent genes.[3,10]

The aim of this study is to determine the relation between sperm functions and the level mRNA expression of NRF2. Therefore, the level mRNA expression of NRF2 in ejaculated spermatozoa and the activity of SOD in seminal plasma were measured, and those values were compared to control group. These results can be helpful in understanding the molecular basis of defective sperm function in humans.

MATERIAL AND METHODS

STUDY SUBJECTS

This study was approved by Necmettin Erbakan University Meram Medical Faculty Ethics Committee with the decision number "2013/119" and this study was supported by Necmettin Erbakan University Research Fund with the project number "131318008". Study subjects were randomly selected from male patients admitted with complaints of infertility who were hospitalized at the in vitro Fertilization Department of Necmettin Erbakan University Meram Faculty of Medicine and Histology-Embryology Department of Selcuk University Faculty of Medicine, between the years of 2013-2015. The study was in accordance with the ethical standards laid down in the 2008 Decleration of Helsinki. All participants in this study were scheduled for an interview after a written informed consent was obtained, and the interviewers collected the subject's data on their medical history, lifestyle, and smoking status with a structured questionnaire. Study subjects were selected with the following inclusion criteria: a) ages were between 25 and 50 years; b) semen quality examination; c) diagnosed as asthenozoospermia or oligoasthenozoospermia and normozoospermia;

and d) without a systemic disease known to affect sperm quality. This study were included 41 patient including 33 asthenozoospermic, 8 oligoasthenozoospermic and 48 normozoospermic males in accordance with spermiyogram analysis evaluated according to the criteria of World Health Organization (WHO-2002). While the mean age of the control group were 31.0 ± 4.21 years, varying from 27 to 43 years, the mean age of the patient group were 32.8 ± 4.78 years, varying from 26 to 43 years.

SEMEN COLLECTION AND GROUPING

Semen samples were collected from patients in sterile containers by masturbation after 3–4 days of sexual abstinence. Samples were allowed to liquefy for at least 30 min at room temperature. Analysis of semen volume, pH, sperm concentration, vitality, immotility, progressive motility, non-progressive motility and abnormal morphology were carried out according to World Health Organization (WHO-2002) guidelines. The remaining semen samples were centrifuged at 500×g for 10 min, and seminal plasma stored at -80 °C until analysis. Cell pellets were frozen respectively at -20 °C 30 min, at -80 °C overnight and immediately thereafter in liquid nitrogen at -196 °C in cryoprotectant agents (DMEM medium supplemented with DMSO) until analyzed.

SUPEROXIDE DISMUTASE ACTIVITY ASSAY

The activity of SOD which is an important antioxidant enzyme was assayed using Superoxide Dismutase Activity Colorimetric Assay kit (Abcam, ab65354) in seminal plasma according to the manufacturer's instruction. GEN5 software of microplate reader system (ELx-800, Biotek) was used for this assay. The inhibition activity of SOD (inhibition rate %) determined by a colorimetric method and read the absorbance at 450 nm using a microplate reader (Abcam, ab65354).

RNA EXTRACTION AND FIRST STRAND CDNA SYNTHESIS

The cell pellet containing sperm were taken from liquid nitrogen and dissolved rapidly within a few minutes at 37 °C. The cell pellet containing sperm was taken to a centrifuge tube and added onto 2 ml of DMEM medium (Sigma-Aldrich, D6429) and it was centrifuged at 500×g for 5 min. Total RNA was isolated from the sperm pellet using RNeasy Lipid Tissue Mini kit (Qiagene, Germany) according to its protocol. The RNA concentration was measured using a NanoDrop spectrophotometer (Thermo Scientific, NanoDrop 2000 UV-Vis spectrophotometer) and stored at -80 °C until preparation of complementary DNA (cDNA).

QUANTITATIVE REAL-TIME REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION

All RNA was reverse transcribed into cDNA using RT² HT First Strand kit (Qiagene, USA) in the thermal cycler (MyCycler Thermal Cycler, BIO RAD) and following qPCR was performed using RT² qPCR Primer Assay kit (Qiagene, USA) according to the manufacturer's instructions. cDNA was used for amplification of NRF2 with the following primer: human NRF2 (PPH06070A-200). Final expression was normalized to the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH): human GAPDH (PPH00150F-200). Data were analyzed using 'Rotor-Gene Q Series Software, QIAGEN' (Rotor-Gene Q, QIAGEN). 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 scale of the amplification curves. Each reaction was analyzed once and CT values were recorded. The ΔCT was calculated to represent the levels of NRF2 antioxidant gene after normalization to that of GAPDH, where $\Delta CT = (C_T$, NRF2 antioxidant genes-C_T, GAPDH).

STATISTICAL ANALYSIS

Levels of NRF2 mRNA expression (ΔCT) and SOD enzyme activities (inhibition rate %) are expressed as mean ± standart deviations. All data were analyzed using a statistical software package (SPSS for Windows, version 20.0, SPSS Inc., USA). The comparison of numerical variables was performed using non-parametric Mann-Whitney U test due to non-normal distribution. Correlation analysis was performed using Spearman's Rho correlation test. A p-value of less than 0.05 was considered to be statistically significant.

RESULTS

SEMEN PARAMETERS OF STUDY SUBJECTS

The mean age, semen parameters of 48 controls and 41 patients are given in Table 1. These data showed that there was no significant differences in mean age between the two study groups (p=0.061). However, basic semen quality parameters including sperm concentration (50.3×10^6/ml and 86.7×10^6/ml), vitality (77% and 90%), and progressive motility (32% and 60%) were significantly lower in patients than in controls (p<0.001), whereas sperm immotility (52% and 24%) and abnormal morphology (90% and 84%) were significantly higher in patients than in controls (respectively; p<0.001, p=0.001). Moreover, there was no significant differences in non-progressive motility (14% and 14%) between the two study groups (p=0.630) (Table 1).

mRNA EXPRESSION LEVEL OF NRF2 ANTIOXIDANT GENE IN SPERMATOZOA

Quantitative real-time reverse transcriptase polymerase chain reaction analysis was performed to determine mRNA expression level of NRF2 antioxidant gene in spermatozoa from men with different sperm motility. These data showed that there was no significant difference in mRNA expression level of NRF2 antioxidant gene between the two study groups (p=0.633) (Table 1, Figure 1).

TABLE 1: Comparison of age, semen parameters, antioxidant gene expression in spermatozoa and seminal plasma SOD activity in the controls and patients.

	Controls (n=48)[a]	Patients (n=41)[a]	p value[b]
Age (yrs)	31.0±4.21	32.8±4.78	0.061
Concentration (10^6/ml)	86.7±54.8	50.3±50.5	0.000
Vitality (%)	90±5	77±13	0.000
Progressive motility (%)	60±7	32±12	0.000
Non-progressive motility (%)	14±6	14±7	0.630*
İmmotility (%)	24±9	52±16	0.000
Abnormal morphology (%)	84±9	90±6	0.001
NRF2[c]	5.34±2.67	5.94±3.20	0.633*
Seminal SOD activity (%)	100.2±20.4	99.6±34.4	0.502

[a] Data are presented as mean ± standart deviation.
[b] p value was calculated by Independent Sample Mann-Whitney U test.
[c] ΔCT= (C_T, antioxidant gene - C_T, GAPDH)
* no statistically significant.

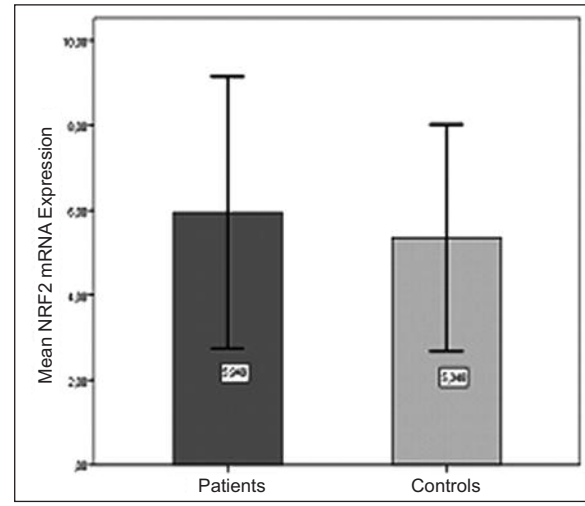

FIGURE 1: mRNA expression level of NRF2 antioxidant gene in the controls and patients.

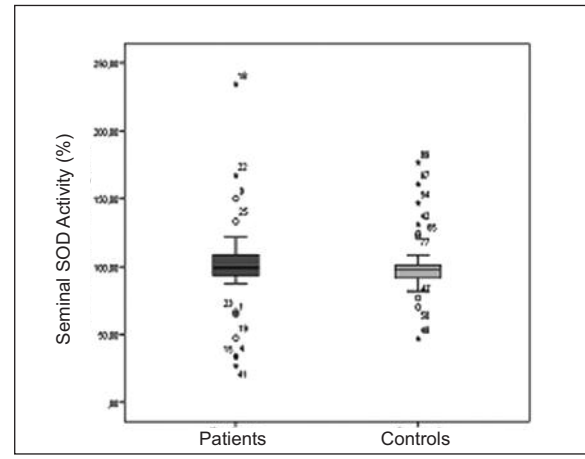

FIGURE 2: Seminal plasma SOD activity of controls and patients.

SOD ACTIVITY IN SEMINAL PLAZMA

Seminal plasma SOD activity in the patients and controls is presented in Figure 2. The mean values of SOD activity in patients and controls were 99,6 ± 34,4% and 100,2 ± 20,4% respectively (Table 1). These data showed that there was no significant difference in seminal plasma SOD activity between the two study groups (p=0.502) (Table 1).

CORRELATIONS BETWEEN NRF2 mRNA EXPRESSION LEVEL AND SPERM QUALITY, SEMINAL SOD ACTIVITY

Spearman correlation analysis showed that the NRF2 mRNA expression was not correlated with sperm parameters, including concentration, pro-

gressive motility, non-progressive motility, immotility, abnormal morphology (p>0.05) (Table 2). But it was also found to be associated with vitality in only patients group (p=0.043) (Table 2). Although the significance value was 0.043, this negative correlation was not considered statistically significant because of its low level (31%). The level of NRF2 mRNA was not also found to be associated with seminal SOD activity (p=0.533) (Table 2).

CORRELATIONS BETWEEN SEMINAL SOD ACTIVITY AND SPERM QUALITY

Spearman correlation analysis showed that the seminal SOD activity was not correlated with sperm parameters, including concentration, vitality, progressive motility, non-progressive motility, immotility (p>0.05) (Table 3). But it was also found to be associated with abnormal morphology in only patients group (p=0.041) (Table 3). Although the significance value was 0.041, this negative correlation was not considered statistically significant because of its low level (32%).

TABLE 2: Correlations between NRF2 mRNA level and sperm quality, seminal SOD activity.

	Controls (n=48)		Patients (n=41)	
	Correlation coefficient[a]	p value	Correlation coefficient[a]	p value
Sperm concentration	0.003	0.984	0.013	0.934
Sperm vitality	0.121	0.412	-0.318	0.043
Sperm progressive motility	-0.111	0.452	0.055	0.734
Sperm non-progressive motility	-0.106	0.475	0.075	0.640
Sperm immotility	0.025	0.866	-0.052	0.747
Sperm abnormal morphology	-0.036	0.810	-0.002	0.988
Seminal SOD activity	0.054	0.713	0.100	0.533

[a] Correlation coefficient was calculated using the Spearman correlation test.

TABLE 3: Correlations between seminal SOD activity and sperm quality.

	Controls (n=48)		Patients (n=41)	
	Correlation coefficient[a]	p value	Correlation coefficient[a]	p value
Sperm concentration	0.125	0.399	0.262	0.097
Sperm vitality	-0.188	0.200	0.051	0.753
Sperm progressive motility	0.093	0.531	-0.029	0.858
Sperm non-progressive motility	0.094	0.524	-0.012	0.941
Sperm immotility	-0.174	0.237	0.051	0.749
Sperm abnormal morphology	0.221	0.132	-0.325	0.041

[a] Correlation coefficient was calculated using the Spearman correlation test.

DISCUSSION

Oxidative stress related with disfunction of sperm motility, high rate of reduction in sperm vitality and low spermatogenesis are common reasons of male infertility.[4,5,12] NRF2 regulates basal and inducible transcription of genes that have antioxidant response elements (AREs) in their promoters and encoding enzymes important for protection against ROS.[3,13] It has also been reported that the detection of mRNA in human ejaculated spermatozoa can be used for diagnosis.[14,15] Therefore, the analysis of sperm mRNA profiles in ejaculated spermatozoa may be useful as a prognosis value for fertilization and a markers for predicting male infertility.[16,17] A previous study, aiming to determine the functional role of mRNA transcripts in bull spermatozoa, supports the result that sperm RNA profiling is suitable for molecular diagnosis of male gamete quality.[18]

There are some studies researching the levels of different transcripts of mRNA encoding molecules containing nuclear condensation, capasity and sperm function of spermatozoa.[19,20] In these studies, it has been submitted that the quality of sperm can be related with its mRNA distribution. Also in same other studies, it has been concluded that human semen with low quality is related with abnormal sperm mRNA content for certain genes.[16,17,21]

Polymorphisms in NRF2 gene were searched to insight into the relationship between defects in the NRF2 gene and human diseases. These results provided a molecular basis for the genetic analysis of the NRF2 gene and furthermore available lines of evidence show that it is worth to investigate the effect of NRF2 in other oxidative stress associated disease prognosis. However, the effects of NRF2 on male reproduction function have not been searched.[22,23] Nakamura et al. established the first study showing the role of NRF2 in male fertility. In this study, it was demonstrated that, the testicular spermatogenesis is highly susceptible to

disruption by chronic oxidative stress in NRF2 knockout mice and NRF2 plays a critical role to prevent oxidative disruption of spermatogenesis. Moreover, it has been shown that knockout of NRF2 gene in mice causes the reduction in sperm concentration and motility, and the fertility is decreased in the NRF2 knockout mice as compared to wild-type and heterozygot littermates. Thus, these results have been reported as proof of diminishing fertility.[3]

In our study, the level of mRNA expression of the antioxidant gene NRF2 in spermatozoa of men with low sperm motility has no significant difference as compared to control group (p=0.633) (Table 1, Figure 1). So this result is not compatible with previous studies suggesting that the NRF2 gene plays an important role in human spermatogenesis. Besides we demonstrated that the levels of mRNA expression of the antioxidant gene NRF2 in spermatozoa of male cases with different sperm motility were not associated with both basic sperm parameters such as concentration, vitality, immotility and seminal plasma SOD activity (respectively; p>0.05, p=0.533) (Table 2).

In the NRF2$^{-/-}$ mice, it has been reported that the expression of many antioxidant enzyme like SOD and CAT is decreased.[24] Moreover, it is thought that the high levels of SOD enzyme are relation with impaired sperm function.[8] It has been reported different results of comparisons between the seminal plasma SOD activity of patient and control group in some studies.[25,26] In this study, it has been observed that the seminal plasma SOD activity of patient group has no significant difference as compared to control group (p=0.502) (Table 1, Figure 2). SOD is one of the most important antioxidant enzymes in seminal plasma.[27] Moreover there are some studies showing also significant or unsignificant results of correlation between the SOD activity in seminal plasma and the basic sperm quality parameters.[26,28-31] There was no significant relation between SOD activity in seminal plasma and basic sperm quality parameters such as concentration, vitality, immotility in our study (p>0.05) (Table 3).

As a conclusion, this study expressed that the level of mRNA expression of the antioxidant gene NRF2 in spermatozoa and seminal plasma SOD activity have no significant difference between the patient and control groups. Moreover, there is no significant relation between the level of mRNA expression of the antioxidant gene NRF2 and sperm quality parameters. These results show that there is no relation between the antioxidant level of NRF2 and defective sperm function. Furthermore NRF2 is not enough alone to be used as a marker to determine both the defects related with ROS in sperm functions and the etiology of male infertility. Also the analysis of SOD in this study is not enough to use as a marker for determining sperm fertilization potential. Since SOD enzymes are known as antioxidant enzymes regulated by NRF2, it is thought that no difference in the level of NRF2 expression could be expected in males having no difference in SOD activity. It is also concluded that further studies should be done in this subject with larger population since our study has a small sample of case and control groups.

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