Evaluation of ROS-TAC Score and DNA Damage in Fertile Normozoospermic and Infertile Asthenozoospermic Males

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Purpose: The aim of study was to evaluate reactive oxygen species (ROS), total antioxidant capacity (TAC) and ROS-TAC score as indicator for oxidative stress status as well as 8-hydrodeoxyguanosine (8-OHdG) levels as a marker for DNA damage in the seminal plasma of asthenozoospermia patients compared to normozoospermia samples.

Materials and Methods: The semen samples of 28 fertile normozoospermic donors and 25 infertile men with asthenozoospermia were analyzed according to World Health Organization (WHO) criteria. ROS production was measured in neat semen samples by the chemiluminescent assay. Plasma levels of TAC was measured by commercially available colorimetric assays. The levels of DNA oxidative damage were measured by seminal plasma levels of 8-OHdG using ELISA method. ROS-TAC score was measured using principal component analysis.

Results: Asthenozoospermic men had a higher ROS levels compared to the normozoospermic men (P = .01). However, no significant difference was observed in TAC levels between the groups. ROS-TAC score in asthenozoospermic men was lower than normozoospermic men (P = .02). The levels of 8-OHdG in the asthenozoospermic men were higher than normozoospermic men (P = .01).

Conclusion: The present study demonstrated a decrease in ROS-TAC score and, a high DNA damage in asthenozoospermia compared to normozoospermia. ROS-TAC score can predict the oxidative damage of semen samples of astenozoospermic infertile males.

Keywords: asthenozoospermia; DNA damage; reactive oxygen species; ROS-TAC score; total antioxidant capacity; 8-OHdG.

INTRODUCTION

Asthenozoospermia is one of the common causes of male infertility which is characterized by poor sperm motility because of various etiology such as physiological, anatomical, medical, genetic and dietary factors [1,2]. Physiological amount of reactive oxygen species (ROS) is required for motility promotion, hyperactivation, capacitation, acrosome reaction, nuclear condensation as well as gamete fusion[3-5]. Nevertheless, ROS in pathological levels can damage sperm's function and male fertility by reduction in sperm motility mostly through depletion of intracellular ATP and lipid peroxidation of plasma membrane[6,7].

Excessive concentrations of ROS in semen can be scavenged by the cumulative sum of the enzymatic and non-enzymatic antioxidants called total antioxidant capacity (TAC)[8]. Some studies showed a positive correlation between TAC levels and normal semen parameters [9,10]. When generation of ROS exceeds, the balance between ROS generation and TAC could be distributed, and consequently, oxidative stress (OS) would be occurred. ROS-TAC score as a marker of seminal OS is calculated from seminal ROS level and seminal plasma TAC values. So, ROS-TAC score calculation, over the use of only ROS or TAC, is recommended for prediction of male fertility potential as well as differentiation between fertile and infertile patients. ROS-TAC score shows OS status in the infertile men. In more levels of ROS-TAC score, the possibility of OS is lower[11,12].

Available evidence suggests that OS leads to DNA

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Received November 2016 & Accepted January 2017

Table 1. Sperm parameters in normozoospermic and asthenozoospermic men.

Parameter[a]	Group		
	Normozoospermia (n=28)	Asthenozoospermia (n=25)	*P*
Sperm concentration (10^6/ mL)	47.24 ± 4.29	39.23 ± 4.36	.2
Motility (%)	62.1 ± 3.05	21.18 ± 4.84	< .0001
Morphology (%)	11.47 ± 2.89	7.52 ± 1.81	.2
leukocyte (10^6/ mL)	0.57 ± 0.08	0.61 ± 0.09	NS

Abbreviation:ns, non significant
[a]Data are presented as Mean ± SE using the independent *T* test.

damage, lipid peroxidation, poor sperm function, and difficulties in fertilizing potential of spermatozoa[13]. Oxidative damage to DNA can occur in the form of base modification, deletions, DNA cross-links, DNA strand breaks, frame shifts or with rearrangement of chromosomes[13]. So, the oxidized base, 8-hydrodeoxyguanosine (8-OHdG) is mostly considered as a marker of OS damage to DNA[14,15]. Some studies have shown that high 8-OHdG levels in sperm are associated with male infertility[2,16-18]. Besides, 8-OHdG levels are correlated with sperm motility and morphology[19].
Because of multifactorial nature of male infertility and low prediction capacity of the spermogram, a more powerful test based on OS measurement is required for assessing male factor infertility as well as basic semen analysis[20-23]. Since, ROS assays to evaluate male factor infertility have sensitivity and specificity of 68.8% and 93.8%, respectively[23], therefore, the aim of study was to evaluate OS status by measurement of seminal ROS, TAC levels and ROS-TAC score as well as DNA damage by evaluating 8OHdG levels in seminal plasma of asthenozoospermia samples compared to normozoospermia ones.

MATERILAS AND METHODS

Study Population

The Ethical Committee of Tehran University of Medical Sciences approved the study and informed consent was obtained from all subjects. 28 fertile normozoospermic donors and 25 infertile asthenozoospermic (sperm motility less than %35) men aged 25-35 years, who recruited at treatment center of Omid clinic were enrolled. All of the participants underwent semen analysis. The infertile group was the people who visited the clinic for infertility problem. The fertile group was chosen among the ones who visited the clinic for embryo donation or sex selection. Fertility of the normozoospermic donors was confirmed by the history of at least one child in the last two years and normal sperm parameters according to World Health Organization (WHO) criteria[24].

Procedures

Ejaculates were obtained by masturbation following 48 to 72 hours of sexual abstinence. After semen liquefaction (30 min at 37 °C), concentration (haemocytometer), motility and morphology of sperm (Papanicolaou staining method) as well as semen volume were determined according to WHO manual[24]. The exclusion criteria were leukocytospermia (leukocyte concentration greater than 1×10^6/ml), men with history of smoking, excessive alcohol consumption, endocrine disorders such as diabetes, drug intake such as vitamins supplements, carotene, ascorbate and tocopherol or minerals such as selenium, zinc, and those with varicocele.
An aliquot of well-liquefied neat semen was taken for immediate ROS evaluation by chemiluminescence assay. The remaining portion of neat semen was centrifuged (500g× 10 min) to separate clear seminal plasma from spermatozoa. Then, stored at -80°C for the measurement of the TAC and 8-OHdG analysis.

Measurement of Seminal ROS

ROS production was measured in neat semen samples by chemiluminescence assay. 2 µl of 5 mM freshly prepared solution of luminol (5-amino-2,3-dihy-

Table 2. Comparison of oxidative stress indices (ROS, TAC, and ROS-TAC scores) between normozoospermic and asthenozoospermic men.

Variable[a]	Group		
	Normozoospermia (n=28)	Asthenozoospermia (n=25)	*P*
ROS (RLU/20*10^6)	162.89 ± 41.57	588.22 ± 152.56	.01
TAC (µM)	796.38 ± 86.45	996.34 ± 62.77	.06
ROS-TAC	50 ± 2.95	39.75 ± 3.26	.02

Abbreviation:ns, not significant
[a] Values are mean ± SE. Independent Student's *t*-test was used for the analysis

Table 3. Correlations between ROS-TAC score, ROS and 8OHdG with sperm parameters.

	Sperm concentration		Motility		Morphology		8OHdG		ROS-TAC score		ROS	
Variable	**r**	**P**	**r**	**P**	**r**	**P**	**r**	**P**	**r**	**P**	**r**	**P**
ROS-TAC score	0.2	.1	0.18	.3	0.13	.4	0.22	.1	-	-	-0.5	< .0001
ROS	-0.3	.05	-0.33	.05	-0.17	.3	-0.28	.07	-0.5	< .0001	-	-
8OHdG	0.28	.09	-0.12	.4	0.19	.2	-	-	0.22	.17	0.28	.07

dro-1,4-phthalazinedione; Sigma Chemical Co.,St. Louis, MO) in dimethyl sulfoxide was added to 80 µl of the liquefied neat semen. Chemiluminescence was measured integrally for 15 minutes using a luminometer (LUmo Luminometer, Autobio Labtec Instruments Co. Ltd., Zhengzhou, China). Number of ROS production was expressed as relative light units (RLU) per minute per 20×10^6 spermatozoa (RLU/min/20×10^6)[23,25].

Total Antioxidant Assay

An antioxidant assay kit (Dianbioassay Co. Iran) was applied to evaluate TAC in the seminal plasma samples. Aliquots of the seminal plasma stored at -80°C were thawed at room temperature and assessed according to manufacturer's instructions. Each seminal plasma was diluted 1:10 with deionized water. This method is based on the ability of seminal plasma antioxidants to inhibit oxidation of the more stable ABTS (2,20-Azino-di-[3 ethylbenzthiazoline sulphonate]) to ABTS+. Briefly, 20 µl of sample or standard was added to 180 µl of reagent I and the first absorbance was read quickly at 660 nm. After adding 20 µl of reagent II and incubation in dark place during 10 minutes, the second absorbance was read at 660 nm. Then, the difference between the first and second absorbance was determined and values of seminal plasma TAC (µM) were calculated using a standard curve.

ROS-TAC score Calculation

ROS-TAC score was calculated using principal component analysis (PCA) in order to obtain standard index of oxidative stress[12]. ROS-TAC score minimizes the variance of the individual variables of OS marker (ROS alone or TAC alone)[13]. Briefly, ROS and TAC levels were normalized to the same distribution. Then, the ROS-TAC score was calculated according to linear combinations which was yielded by PCA that is described by Mahfouz et al[12].

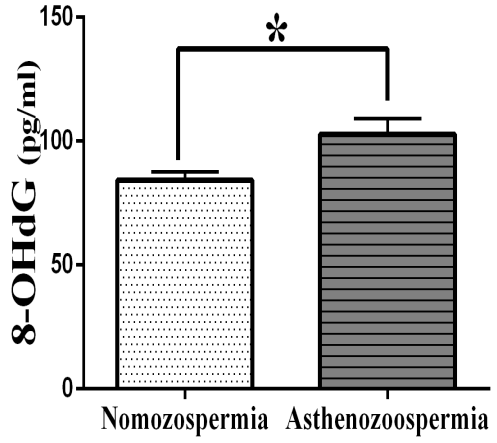

Figure 1. Seminal plasma concentration of 8-OHdG in normozoospermic and asthenozoospermic men. Results are expressed as mean ± SE. *$P < 0.05$.

8-OHdG assay

The levels of 8-OHdG (pg/ml) in seminal plasma were measured using a competitive ELISA kit (Cayman chemical, Cat# 589320, Ann Arbor, MI, USA) according to the manufacturer's protocol. After reading at 450 nm, the concentration of each sample was determined using the equation obtained from the standard curve.

Statistical Analysis

Results were expressed as mean ± SE. Variables were compared using independent Student's t-test among the groups. The linear correlation between two variables were calculated using the Pearson correlation coefficients. Statistical significance was defined as $P \leq .05$.

RESULTS

Sperm parameters according to the groups are presented in **Table 1**. Semen samples in both groups had a concentration and morphology score of more than 15×10^6 cells/ml and 4%, respectively. Total motility was significantly lower in the asthenozoospermic men compared to the normozoospermic men ($P < .001$). According to microscopic observation, samples containing more than 1×10^6 leukocyte per milliliter semen were excluded to avoid a potential source of ROS generation.

Oxidative Stress Detection in Neat Semen

Seminal levels of ROS, seminal plasma TAC levels and calculated ROS-TAC score in the study groups are illustrated in **Table 2**. Asthenozoospermic men had a significantly higher ROS levels compared to normozoospermic men ($P = .01$). However, no significant difference was detected in seminal plasma levels of TAC among the groups ($P = .06$). ROS-TAC score in asthenozoospermic men was significantly lower than normozoospermic men ($P = .02$).

8-OHdG Assay

The seminal plasma levels of 8-OHdG were 84.36 ± 3.29 and 102.82 ± 6.24 (pg/mL) in the normozoospermic and asthenozoospermic men, respectively (**Figure 1**). The levels of 8-OHdG were found to be significantly higher in the asthenozoospermic men compared to the normozoospermic men ($P = .01$).

Correlation Analysis

Correlations of ROS-TAC score, ROS and 8-OHdG with semen parameters in total population are shown in

Table 3. ROS levels negatively correlated with motility (r = -0.33, *P* = .05) and sperm concentration (r = -0.3, *P* = .05). In the present study, no significant correlation was demonstrated between ROS-TAC score and sperm parameter in the groups. Also, no significant relationship was found between the 8-OHdG levels with the ROS-TAC score and ROS.

DISCUSSION

We demonstrated a decrease in ROS-TAC score from asthenozoospermia compared to the normozoospermia. The DNA oxidation levels were also found to be higher in asthenozoospermia.

Asthenozoospermia is an extremely common cause of male infertility[26]. Even though the sperm motility is dependent on ROS generation in physiological levels and consequently oxygen and ATP consumption, ROS in pathological levels appears to have a significant role in reduction of motility due to ATP depletion and lipid peroxidation of sperm membrane[27-30]. The sperm motility was also found to be lower in asthenozoospermia which was in agreement with the results of previous works[6,31]. An elevation in OS in human spermatozoa with lower percentage of motility and morphology in comparison to proven donors have been reported previously[32,33]. In the current study, also ROS levels were increased in asthenozoospermia compared to normozoospermia which is consistent with previous observations[12,34,35].

Contradictory results have been reported on the seminal plasma TAC levels between infertile and fertile males[9,12,34,36]. In the present study, TAC levels were higher in the asthenozoospermia compared to normozoospermia, but the data were very variable so that statistical significance was not achieved. One possible explanation is that the seminal TAC in the initial OS occurrence attempts to scavenge the elevating levels of ROS and therefore the levels of TAC might not be decreased[6,12,37]. Some studies have shown a positive correlation between high seminal ROS levels and lower percentage of motile sperm in infertile males[12,34,35]. Indeed, a majority of ROS production seen in populations of impaired sperm motility is related to the onset of OS which is inconformity with the current study that is probably because of no significant decrease in TAC levels of asthenozoospermia.

In the present study, ROS levels were elevated in asthenozoospermia, however; there was no sufficient increase in TAC levels to compensate OS. Indeed, a balance between ROS generation and TAC is disrupted which may be involved in the pathophysiology of male infertility. Although, ROS and TAC were measured alone like previous studies, calculating of ROS-TAC score as an accurate index of OS, gives more valuable information about male infertility[12,34]. We found a significant lower ROS-TAC score in asthenozoospermia compared to normozoospermia which could lead to OS in asthenozoospermia. According to our result, the occurrence of OS in infertile asthenozoospermic men is more likely affected by increase of ROS[38]. Therefore, the impairment in sperm-fertilizing ability after different treatments may be due to the factors not tested during routine semen analysis, such as OS status. In agreement with our results, Mahfouz et al. revealed that infertile men with high seminal ROS levels have a significant lower ROS-TAC score compared to the men with physiological seminal ROS levels. However, there was no any significant decrease in TAC levels of infertile men with high seminal ROS levels[12]. Therefore, it can be concluded that ROS-TAC score is a more accurate and valid marker of OS than ROS or TAC values alone in male infertility. However, to date there are still a few studies in the field of male infertility which investigated ROS-TAC score.

OS may have negative effects on sperm function through impairment in membrane fluidity and integrity, and DNA integrity[39,40]. In the present study, the 8-OHdG levels of sperm DNA showed a significant increase in asthenozoospermia compared to normozoospermia which is in line with the previous reports [16,33]. It seems that OS, by inducing damage to the sperm DNA, decreases chance of successful fertilization and embryo development after IVF and ICSI[41].

In conclusion, the present study demonstrated a decrease in ROS-TAC score and, a high DNA damage in asthenozoospermia compared to normozoospermia. ROS-TAC score can predict the oxidative damage of semen samples of asthenozoospermic infertile males.

ACKNOWLEDGEMENT

We thank the Omid Clinic, particularly Dr. Saeidi for providing samples. This study was funded by the Tehran University of Medical Sciences, Tehran, Iran (Grant numbers 21660 and 30756).

CONFLICT OF INTEREST

The authors report no conflict on interest.

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