The Evaluation of Total Antioxidant and Oxidant Response in Seminal Plasma in Fertile and Infertile Men Using a Novel Automated Method

Fertil ve İnfertil Erkeklerde Yeni Bir Otomatik Metotla Seminal Plazmada Total Oksidan ve Antioksidan Yanıtın Değerlendirilmesi

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ABSTRACT Objective: Oxidative stress has been implicated in the pathogenesis of male infertility. In this study, we aimed to measure the antioxidant capacity in seminal plasma in fertile and infertile men using a recently developed automated measurement method. **Material and Methods:** The study group included samples of 55 males from infertile couples, and 16 healthy volunteers served as the control group. All samples had normal sperm parameters. Seminal total antioxidant response (TAR) and total oxidant status (TOS) were determined using colorimetric methods. Oxidative stress index (OSI) was calculated as [(TOS/TAR) ×100]. Free sulphydryl (SH) groups of semen samples were measured spectrophotometrically. **Results:** Seminal TAR was significantly lower and seminal TOS, OSI and SH activities were significantly higher in infertile group as compared with fertile donors. There were positive correlations between TAR and sperm parameters such as +4 motility ($r= 0.386$, $p< 0.01$), and morphology ($r= 0.383$, $p< 0.01$), whereas sperm concentrations ($r= -0.333$, $p< 0.01$) correlated negatively with TAR. In addition, TOS and OSI were, negatively correlated with sperm parameters such as motility, +4 motility and morphology ($p< 0.01$, for all). **Conclusion:** Increased oxidant status and decreased antioxidant response and the correlation between these parameters and sperm functions implicated that oxidative stress might play an important role in male infertility. We found decreased total antioxidant response in infertile patients using a simple, rapid and reliable automated colorimetric assay, which may suitable for use in any routine andrology laboratory.

Key Words: Infertility; oxidative stress; semen

ÖZET Amaç: Erkek infertilitesinin patogenezinde oksidatif stres de dahil edilmektedir. Bu çalışmada, fertil ve infertil erkeklerin seminal sıvılarındaki antioksidan kapasiteyi yeni geliştirilmiş otomatik bir metotla ölçmeyi ve değerlenmeyi amaçladık. **Gereç ve Yöntemler:** Çalışma grubu infertil çiftlerden 55 erkek hastayı içerirken, sperm parametreleri normal olan 16 sağlıklı gönüllü de kontrol grubunu oluşturdu. Semendeki total antioksidan yanıt (TAR) ve total oksidan durum (TOS) kolorimetrik yöntemlerle belirlendi. Oksidatif stres indeksi (OSI); [(TOS/TAR) ×100] şeklinde hesaplandı. Semen örneklerindeki serbest sülfidril grupları spektrofotometrik olarak ölçüldü. **Bulgular:** Erkek infertilitesi olan grupta fertil gönüllülerle karşılaştırıldığında semendeki TAR anlamlı derecede düşük, TOS, OSI ve sülfidril aktiviteleri ise anlamlı derecede yüksekti. TAR ve +4 motilite ($r= 0.386$, $p< 0.01$) ve morfoloji ($r= 0.383$, $p< 0.01$) gibi sperm parametreleri arasında doğru orantı; sperm konsantrasyonu arasında ise ters orantı ($r= -0.333$, $p< 0.01$) mevcuttu. Ayrıca, TOS ve OSI ile motilite, +4 motilite ve morfoloji gibi sperm parametreleri ($p< 0.01$, hepsi için) arasında da ters orantı bulundu. **Sonuç:** Artmış oksidatif stres, azalmış antioksidan yanıt ve bu parametrelerle sperm fonksiyonları arasındaki ilişki göstermektedir ki erkek infertilitesinde oksidatif stres önemli rol oynayabilmektedir. Biz infertil hastalarda rutin androloji laboratuarında kullanılabilecek basit, hızlı ve güvenilir bir otomatik kolorimetrik tahlil yöntemi ile antioksidan cevapta azalma tespit ettik.

Anahtar Kelimeler: Kısırlık; oksidatif stres; semen

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Male gender has been considered a major contributory factor to infertility. Along with the conventional causes for male infertility such as varicocele, cryptorchidism, infections, obstructive lesions, cystic fibrosis, trauma and tumors, a new and important cause has been identified: oxidative stress (OS) is a result of the imbalance between reactive oxygen species (ROS) and antioxidants in the body which can lead to sperm damage, deformity and eventually male infertility.[1] ROS are essential to mammalian sperm functions including maturation, capacitation, acrosome reaction, sperm-oocyte fusion and the stimulation of hyper-activated sperm motility.[2,3] On the other hand, excessive generation of ROS can impair normal sperm function by peroxidation of unsaturated fatty acids in membrane of spermatozoa and by DNA fragmentation.[4] Various recent studies have demonstrated that oxidative stress and oxidative damage increased significantly in spermatozoa with declined motility and the antioxidant capacities in the spermatozoa and seminal plasma were lower in males who had infertility.[4-7] Conversely Siciliano et al. showed that non-enzymatic antioxidant capacities of seminal plasma were not altered the asthenozoospermic specimens.[8]

The cellular damage in the semen is the result of an improper balance between ROS generation and scavenging activities. Therefore, numerous antioxidants such as vitamin C, vitamin E, glutathione and coenzyme Q10 have proven to have beneficial effects in treating male infertility.[9]

Measurement of total antioxidant response (TAR) within semen can be conducted in a variety of ways.[10] These include enhanced chemiluminescence's (ECL) assays, spectrophotometric methods, fluorometric methods and electrochemical methods.[4] However; there is yet no accepted "gold standard" reference method.[11]

In this study, we aimed to measure both the levels of free sulphydryl (SH) groups and the TAR values in seminal plasma from infertile men to evaluate their antioxidant status using a novel automated method.[11] As a reciprocal measure, the TOS was also measured in the plasma samples of the same individuals. The percent ratio of the total plasma peroxide level to the plasma TAR level was regarded as the oxidative stress index (OSI).[12]

MATERIAL AND METHODS

SELECTION OF SUBJECTS

The study group included samples of 55 males from infertile couples, treated in Irenbe IVF center. Sixteen healthy volunteers served as the control group where all samples had normal sperm parameters. All men gave informed consent for participating in the study, which had been approved by the local Ethics Committee (Izmir Tepecik Training and Research Hospital local Ethic Committee: 30.03.2007-67/11). None of the patients had received any medication or vitamin supplementation. Instances of leukocytospermia and viscous semen samples were excluded from this study.

SEMEN COLLECTION AND PREPARATION

Semen specimens were collected by masturbation after 48 to 72 hours of sexual abstinence. The specimens underwent complete liquefaction at 37°C for 20 minutes. All semen samples were counted in a Mackler counting chamber (Sefi Medical Instruments, Rehovot, Israel). The samples were classified according to the criteria of the World Health Organization Laboratory Manuel, 4th edition.[13] The motility of sperm has been classified as +1, +2, +3, and +4 motile. Morphology smears were scored by using Kruger's strict criteria.[14] All samples were centrifuged at 1000 × g for 10 minutes. Clear seminal plasma were stored at -80°C until the analysis and transferred to Harran University with the dry ice. All the experimental studies were conducted in the next day in Harran University Faculty of Medicine, Department of Clinical Biochemistry laboratories.

MEASUREMENT OF THE TOTAL ANTIOXIDANT RESPONSE OF SEMINAL PLASMA

The total antioxidant status of the seminal plasma was measured using a novel automated colorimetric method for the TAR developed by Erel.[15] The method was applied to an automated analyzer (Abbott, Illinois, USA). In this method, the hydroxyl

radical, the most potent biological radical, is produced by Fenton reaction and it reacts with the colorless substrate *O*-dianisidine to produce the dianisyl radical, which is bright yellowish-brown in color. Upon the addition of a plasma sample, the oxidative reactions initiated by the hydroxyl radicals present in the reaction mix are suppressed by the antioxidant components of the seminal plasma, preventing the color change and thereby providing an effective measure of the total antioxidant capacity of the seminal plasma. The assay results were expressed as mmol Trolox eq./L. The assay has got excellent precision values, within and between precision values were lower than 3%.[16]

MEASUREMENT OF THE TOTAL OXIDATION STATUS OF SEMINAL PLASMA (THE TOTAL SEMINAL PLASMA PEROXIDE CONCENTRATION)

The total oxidant status of the plasma was measured using a novel automated colorimetric method for TOS developed by Erel.[17] The method was applied to an automated analyzer (Abbott, Illinois, USA). In this method oxidants present in the sample oxidize the ferrous ion-o-dianisidine complex to ferric ion. The oxidation reaction is enhanced by glycerol molecules, which are abundantly present in the reaction medium. The ferric ion makes a colored complex with xylenol orange in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay was calibrated with hydrogen peroxide and the results were expressed in terms of micromolar hydrogen peroxide equivalent per liter (μmol H_2O_2 Eq/L). The novel assay is linear up to 200 μmol H_2O_2 Equiv./L and its precision value is lower than 3%. The lower detection limit is 1.13 micromol H2O2 Equiv./L.[16]

MEASUREMENT OF FREE SULPHYDRYL GROUPS OF SEMINAL PLASMA

Free sulphydryl groups of semen samples were determined using the Ellman method[18] modified by Hu et al.[19] One ml of buffer containing 0.1 M Tris, 10 mM EDTA, pH 8.2, and 50 μl seminal plasma were added to cuvettes, followed by 50 μl 10mM DTNB in methanol. Blanks were run for each sample as a test, but there was no DTNB in the methanol. Following incubation at room temperature for 15 min, absorbance of sample was read at 412 nm on a Cecil 3000 spectrophotometer. Sample and reagent blanks were subtracted. The concentration of SH was calculated using reduced glutathione as SH group standard and the results were expressed as millimolars (mmol/mL).

CALCULATION OF OXIDATIVE STRESS INDEX OF SEMINAL PLASMA

The percent ratio of the total oxidation status level to the total antioxidant response was accepted as the OSI, an indicator of the degree of oxidative stress.[12] OSI value was calculated with the formula below;

OSI= [(TOS, μmol H_2O_2 equivalent/L)/(TAR, μmol Trolox equivalent/L)] X100

For a detailed understanding of measurements, readers should refer to Erel's articles.[15,17]

STATISTICAL ANALYSIS

Differences between control and male infertility groups were assessed by using Mann Whitney U test and coefficients of correlation were calculated using Spearman's correlation analysis. All hypothesis tests were two-tailed with statistical significance set at $p< 0.05$. The data were expressed as the median (min-max). Statistical computations were calculated using SPSS 11.0 for Windows software (SPSS Inc, Chicago,IL, USA).

RESULTS

Demographic data, semen characteristics, seminal TAR, TOS, OSI, and SH activities in infertile males and fertile donors are summarized in Table 1. There was no significant difference in terms of age between the groups. Seminal TAR was significantly lower and seminal TOS, OSI and SH activities were significantly higher in male factor infertility group as compared with fertile donors (Table 1).

The relationship between seminal TAR, TOS, OSI, and SH activities and semen parameters in the whole group (n= 71) are shown in Table 2. There were positive correlations between TAR and sperm parameters such as +4 motility and morp-

TABLE 1: Demographics, semen characteristics, seminal oxidative and antioxidative parameters of infertile and fertile donors.

	Fertil donors (n= 16)	Infertile donors (n= 55)	P
Age (years)	34.00 (21-43)	32.00(24-61)	0.715
Semen parameters			
Concentrations (×10^6/mL)	42.56 (22-100)	23.80 (1-88)	0.039
Motility (% motile sperm)	55 (44-70)	36 (0-49)	<0.001
Morphology (Kruger's criteria % normal sperm)	16.50 (6-25)	6.00 (0-10)	<0.001
+4 Motility (% bir tık motile sperm)	25 (12-30)	9 (0-15)	<0.001
TAR (mmol Trolox eq. /L)	10.96 (1.59-11.00)	4.72 (0.36-10.98)	<0.001
TOS (μmol H_2O_2 eq./L)	185(14-704)	721(13-1036)	<0.001
OSI (AU)	0.169 (0.1-4.43)	1.54 (0.1-19.50)	0.003
SH (mmol/L)	13.80(2.40-45.00)	20.40(0.10-42.60)	0.005

Values are medians (min-max).

TABLE 2: Correlations between sperm parameters and seminal TAR, TOS, OSI and SH in the wholel group (n= 71).

Semen Parameters	TAR (mmol Trolox eq. /L)	TOS (μmol H_2O_2eq./L)	OSI (AU)	SH (mmol/L)
Concentrations (×106/mL)	r= -0.333	p= 0.837	r= 0.315	p= 0.250
	p= 0.005		p= 0.007	
Motility (% bir tık motile sperm)	p= 0.107	r= -0.320	r= -0.235	p= 0.101
		p= 0.006	p= 0.048	
Morphology (Kruger's criteria % normal sperm)	r= 0.383	r= -0.432	r= -0.412	p= 0.342
	p= 0.001	p< 0.001	p< 0.001	
+4 Motility (% bir tık motile sperm)	r= 0.386	r= -0.418	r= -0.408	p= 0.074
	p= 0.001	p< 0.001	p< 0.001	

Values are Spearman's correlation coefficients (r).

hology (p< 0.01, for all) whereas sperm concentrations correlated negatively with TAR. In addition, there were negative correlations between TOS and OSI with sperm parameters such as motility, +4 motility, and morphology (p< 0.01, for all) in the study.

DISCUSSION

Male factor infertility accounts for 30% to 50% of all infertile couples seeking for infertility management.[20] Evaluation of male gender infertility is becoming more important and informative as new diagnostic techniques and therapeutic options become available.[1,7]

Evidence now suggests that ROS-mediated damage to sperm is a significant contributing pathology in 30-80% of infertile patients.[10] Oxidative stress is a condition that is associated with an imbalance between the production and removal of ROS and free radicals.[1,3,7] All cellular components including lipids, proteins, nucleic acids, and sugars are potential targets of ROS.[1] Sperm membranes are vulnerable to this type of damage as they contain large amounts of unsaturated fatty acids.[10] ROS-induced peroxidation of the sperm membrane have been correlated with sperm dysfunctions through different mechanism that include lipid peroxidation of plasma membrane and impairment of sperm metabolism, motility, and fertilizing capacity.[21] Total seminal plasma antioxidants constiture the most protective defensive mechanism available to spermatozoa against ROS.

Low seminal total antioxidant capacity has been shown to be related to male infertility.[4,5,20,22,23]

In our study, seminal TAR was significantly lower and seminal TOS and SH activities were significantly higher in infertile patient group as compared with control group (Table 1).

Our findings confirm the results of Koca et al., Fingerova et al., and Mahfouz et al. about TAR activity in infertile men.[4,5,20] Koca et al. evaluated TAR in infertile asthenozoospermic and asthenoteratozoospermic men and compared them with normozoospermic fertile men.[5] Their study showed that asthenozoospermic and asthenoteratozoospermic males have significantly lower TAR value than the control group. Fingerova et al. found that the seminal plasma TAR values in the males from infertile couples were significantly lower than the control group. In addition, they found the highest sperm ROS productions in the samples of 5 infertile males.[4] This fact may be a reflection of the reciprocal relationship between the two extremes of the oxido-reductive balance in the seminal plasma.

In our study the correlations between TAR and sperm parameters such as +4 motility ($r= 0.386$, $p< 0.05$) and morphology ($r= 0.383$, $p< 0.05$) were positive (Table 2).

Khosrowbeygi et al. examined the positive correlation between total antioxidant capacity (TAC) and sperm motility and morphology in asthenozoospermic, asthenoteratozoospermic and oligoasthenoteratozoospermic males.[6] Koca et al.'s study showed that TAC correlated positively with sperm motility.[5] Conversely Siciliano et al. showed that in semen with normal viscosity levels of catalase, SOD, and TAR were not different in asthenozoospermic specimens compared with normozoospermic men.[8]

Different findings from different studies could be related to existence of different motility grades of sperm. We found positive correlation only between TAR and +4 motility.

SH groups in seminal plasma are the most critical targets for free radical attacks.[24] In our study, seminal SH activities were significantly higher in infertile patients. Our findings confirm the results of Zini et al.[25] however contradict the results of Kao et al.,[7] Alkan et al.,[26] and Ebisch et al.[27] Zini et al. demonstrated that sperm SH content correlated positively with sperm DNA denaturation and there were significantly higher levels of sperm DNA denaturation and SH content there were in infertile men.[25] Kao et al. showed that sperm with higher levels of oxidative damage had lower amount of thiols in seminal plasma.[7] Alkan et al. reported that seminal plasma SH groups in infertile patients were significantly lower than those in the control groups.[26] Ebisch et al. found no significant difference in SH groups of seminal plasma between fertile and infertile males.[27] Similar to our results, Lewis et al. observed significantly higher thiol concentrations in spermatozoa of asthenozoospermic men.[28] According to their explanation, this may be due to the contribution of ROS produced by spermatozoa in this group leading to the up-regulation of thiol synthesis in order to protect the spermatozoa from oxidative damage. These authors speculate that the high thiol concentrations caused reduced motility in these spermatozoa because of a decrease in disulphide bonding during sperm maturation in the epididymis.

Use of antioxidants is not routine in clinical practice.[1] Numerous antioxidants such as vitamin C, vitamin E, glutathione, selenium and coenzyme Q10 have proven beneficial effects in treating male infertility.[9,29,30] Nevertheless some studies failed to demonstrate the same benefit.[31-33] Studies suffer from lack of placebo-controlled, double-blind design.[34,35] In addition, investigators design studies on men in whom oxidative stress is not implicated as an infertility factor.[32,33] Routine use of TAR evaluation in andrology laboratory may clarify these contradicted results.

The existing methods for the measurement of TAR are enhanced chemiluminescence (ECL) assays, spectrophotometric methods such as the ferric reducing ability (FRAP) assay, CUPRAC assay or methods based on the formation of the $ABTS^{+}$ radical, fluorometric methods such as the oxygen radical absorbance capacity (ORAC) assay, electrochemical methods like coulometry, voltammetry or electron spin resonance (ESR) assay.[2,4]

The ECL assay is the most commonly used method for measuring TAC in seminal fluid.[36] The measurement of TAC by ECL assay method generally takes approximately 40 to 45 minutes; ORAC assay for measuring TAC has a significantly less reagent cost, it requires a fluorescence detector and

takes more than 70 minutes longer to complete than the colorimetric assay.[37] Although the cost of reagents for the ECL assay (buffers, signal reagent, peroxidase reagent) is comparable to the cost of a colorimetric assay kit, the price of an average luminometer with kinetic setting averages $30.000 compared with $6000 for a simple spectrophotometer.[36] The FRAP assay is simple and inexpensive, however does not measure the SH-group-containing antioxidants.[37]

On the other had, there is yet no accepted, "gold standard'' reference method, but the novel assay reported by Erel has several advantages. It is simple, inexpensive, rapid and can easily be fully automated. Accurate measurements of the total seminal plasma antioxidant response can be obtained in only 10 minutes.[15]

We measured total oxidant levels in seminal plasma in our study with the colorimetric method that was developed by Erel.[17] This technique has many advantages. Various other methods that have been developed for measuring TOS had no accepted reference method. In addition, this method has a final decision concerning the standardizations, the terms and the units.[17] Moreover, the fluorescence, chemiluminescence and ESR methods need sophisticated techniques and, in most routine andrology laboratories, these improved systems are not available.

In this study, we aimed to measure the TAR values in seminal plasma from infertile men to evaluate their antioxidant status using a novel automated method.[11]

In conclusion, detecting seminal plasma TAR levels in andrology laborator, as a routine and rapid test may be useful in the management of infertility.

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REFERENCES

1 Agarwal A, Makker K, Sharma R. Clinical relevance of oxidative stress in male factor infertility: an uptade. Am J Reprod Immunol 2008;59(1):2-11.

2 Lissak A, Wiener-Megnazi Z, Reznick AZ, Shnizer S, Ishai D, Grach B, et al. Oxidative stress indices in seminal plasma, as measured by the thermochemiluminescence assay ,correlate with sperm parameters. Fertil Steril 2004;81(Supple 1):792-7.

3 Sakamoto Y, Ishikawa T, Kondo Y, Yamaguchi K, Fujisawa M.The assessment of oxidative stress in infertile patients with varicocele. BJU Int 2008;101(12):1547-52.

4 Fingerova H, Novotny J, Barborik J, Brezinova J, Svobodova M, Krskova M, et al. Antioxidant capacity of seminal plasma measured by TAS Randox. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 2007;151(1):37-40.

5 Koca Y, Ozdal OL, Celik M, Unal S, Balaban N. Antioxidant activity of seminal plasma in fertile and infertile men. Arc Androl 2003;49(5):355-9.

6 Khosrowbeygi A, Zarghami N. Levels of oxidative stress biomarkers in seminal plasma and their relationship with seminal parameters. BMC Clin Pathol 2007;7:6.

7 Kao SH, Chao HT, Chen HW, Hwang TI, Liao TL, Wei YH. Increase of oxidative stress in human sperm with lower motility. Fertil Steril 2008;89(5):1183-90.

8 Siciliano L, Tarantino P, Longobardi F, Rago V, De Stefano C, Carpino A. Impaired seminal antioxidant capacity in human semen with hyperviscosity or oligoasthenozoospermia. J Androl 2001;22(5):798-803.

9 Sheweita SA, Tilmisany AM, Al-Sawaf H. Mechanisms of male infertility: role of antioxidants. Curr Drug Metab 2005;6(5):495-501.

10 Tremellen K.Oxidative stress and male infertility-a clinical perspective. Hum Reprod Uptade 2008;14(3):243-58.

11 Harma M, Harma M, Erel O. Measurement of the total antioxidant response in preeclampsia with novel automated method.Eur J Obstet Gynecol Reprod Biol 2005;118(1):47-51.

12 Harma M, Harma M, Erel O. Increased oxidative stress in patients with hydatiform mole. Swiss Med Wkly 2003;133(41-42):563-6.

13 World Health Organization. WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction. 4thed. Cambridge: Cambridge University Press; 1999.

14 Kruger TF, Menkveld R, Stander FS, Lombard CJ, Van der Merwe JP, van Zyl JA, et al. Sperm morphologic features as a prognostic factor in in vitro fertilization. Fertil Steril 1986;46(6):1118-23.

15 Erel O. A novel automated method to measure total antioxidant response against potent free radical reactions.Clin Biochem 2004;37(2):112-9.

16 Verit FF, Verit A, Kocyigit A, Ciftci H, Celik H, Koksal M. No increase in sperm DNA damage and seminal oxidative stress in patients with idiopathic infertility. Arch Gynecol Obstet 2006;274(6):339-44.

17 Erel O. A new automated colorimetric method for measuring total oxidant status. Clin Biochem 2005;38(12):1103-11.

18 Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys 1959;82(1):70-7.

19 Hu ML, Louie S, Cross CE, Motchnik P, Halliwell B. Antioxidant protection against hyochlorous acid in human plasma. J Lab Clin Med 1993;121(2):257-62.

20 Mahfouz R, Sharma R, Sharma D, Sabanegh E, Agarwal A. Diagnostic value of the total antioxidant capacity(TAC)in human seminal plasma. Fertil Steril 2009;91(3):805-11.

21 Saleh RA, Agarwal A, Nada EA, El-Tonsy MH, Sharma RK, Meyer A, et al. Negative effects of increased sperm DNA damage in relation to seminal oxidative stress in men with idiopathic and male factor infertility. Fertil Steril 2003;79(Supple 3):1597-605.

22 Lewis SE, Boyle PM, McKinney KA, Young IS, Thompson W. Total antioxidant capacity of seminal plasma is different in fertile and infertile men. Fertil Steril 1995;64(4):868-70.

23 Smith R, Vantman D, Ponce J, Escobar J, Lissi F. Total antioxidant capacity of human seminal plasma.Hum Reprod 1996;11(8):1655-60.

24 Miesel R, Drzyczak PJ, Kurpisz M. Oxidanve stress during the interaction of gametes. BtoL Reprod 1993;49(5):918-23.

25 Zini A, Kamal KM, Phang D. Free thiols in human spermatozoa: correlation with sperm DNA integrity. Urology 2001;58(1):80-4

26 Alkan I, Simsek F, Haklar G, Kervancioglu E, Ozveri H, Yalcin S, et al. Reactive oxygen species production by the spermatozoa of patients with idiopathic infertility: relationship to seminal plasma antioxidants. J Urol 1997;157(1):140-3.

27 Ebisch IM, Peters WH, Thomas CM, Wetzels AM, Peer PG, Steegers-Theunissen RP. Homocysteine, glutathione and related thiols affect fertility parameters in the (sub)fertile couple. Hum Reprod 2006;21(7):1725-33.

28 Lewis SE, Sterling ES, Young IS, Thompson W. Comparison of individual antioxidants of sperm and seminal plasma in fertile and infertile men. Fertil Steril 1997;67(1):142-7.

29 Kessopoulou E, Powers HJ, Sharma KK, Pearson MJ, Russell JM, Cooke ID, et al. A double-blind randomized placebo cross-over controlled trial using the antioxidant vitamin E to treat reactive oxygen species associated male infertility. Fertil Steril 2005;64(4): 825-31.

30 Keskes-Ammar L, Feki-Chakroun N, Rebai T, Sahnoun Z, Ghozzi H, Hammami S, et al. Sperm oxidative stress and the effect of an oral vitamin E and selenium supplement on semen quality in infertile men. Arch Androl 2003;49(2):83-94.

31 Donnelly ET, McClure N, Lewis SE. Antioxidant supplementation in vitro does not improve human sperm motility. Fertil Steril 1999;72(3):484-95.

32 Bolle P, Evandri MG, Saso L. The controversial efficacy of vitamin E for human male infertility. Contraception 2002;65(4):313-5.

33 Comhaire FH, Garem Y, Mahmoud A, Eertmans F, Schoonjans F. Combined conventional/antioxidant''Astaxanthin''treatment for male infertility, double blind, randomized trial. Asian J Androl 2005;7(3):257-62.

34 Agarwal A, Nallella KP, Allamanei SSR, Said TM. Role of antioxidants in treatment of male infertility:an overview of the literature. Reprod Biomed 2004;8(6):616-27.

35 Patel SR, Sigman M. Antioxidant therapy in male infertility. Urol Clin North Am 2008;35(2):319-30.

36 Said TM, Kattal N, Sharma RK, Sikka SC, Thomas AJ Jr, Mascha E, et al. Enhanced chemiluminescence assay vs colorimetric assay for measurement of the total antioxidant capacity of human seminal plasma. J Androl 2003;24(5):676-80.

37 Cao G, Prior RL. Comparison of different analytical methods for assessing total antioxidant capacity of human serum. Clin Chem 1998;44(6 Pt 1):1309-15.