African Journal of Biotechnology Vol. 9(35), pp. 5739-5745, 30 August, 2010
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.429
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

The correlation between total antioxidant capacity and nitric oxide concentration in seminal plasma with sperm DNA damage

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Accepted 11 August, 2010

Sperm DNA quality is important in male fertility. Oxidative stress increases sperm DNA damages. Antioxidants decrease production of free radicals and scavenge them. Nitric oxide (NO) is a free radical which is produced by most cells and has a dual role on cells. Low concentrations of NO is essential in biology and physiology of systems but high level of NO has a detrimental effect on cells. The aim of this study was to determine the role of the nitric oxide concentration and total antioxidant capacity (TAC) in seminal plasma with sperm DNA damage. Semen samples from 45 infertile men and 70 normozospermic men were examined for DNA damage, nitric oxide concentration and TAC. DNA damage was measured by comet assay and nitric oxide concentration was evaluated by Griess assay. TAC was measured in seminal plasma based on the generation of peroxyl radicals from 2,2-azinobis (2-amidino propane) dihydrochlorid (AAPH). Our results show that the means of DNA damage and nitric oxide concentration in infertile men was higher than fertile men. TAC level in infertile men was significantly lower than fertile men. DNA damage was significantly correlated with nitric oxide concentration in infertile men (p = 0.001, r = +0.598) and TAC (p = 0.04, r = - 0.3) in infertile men. In conclusion, sperm DNA damage in infertile men may be induced by nitric oxide-mediated oxidative stress and low levels of TAC.

Key words: Nitric oxide, male infertility, total antioxidant capacity, DNA damage.

INTRODUCTION

The role of sperm DNA quality in male fertility is well documented (Shen and Chia, 1999; Agarwal and Tamer, 2003a; Moustafa et al., 2004). Intact human sperm DNA is not only essential for successful fertilization but is also important in implantation and subsequent embryo development (Agarwal and Allameneni, 2004). A negative correlation between the degree of DNA damage with fertilization, embryo cleavage, implantation, pregnancy and live birth rates, has been observed (Shamsi et al., 2008; Evenson et al., 2002). Also, a higher spontaneous abortion rate was reported when more than 30% of sperms contain fragmented DNA (Evenson et al., 2002).

The most important causes of sperm DNA damages are abnormal chromatin packing, reactive oxygen species (ROS) and disruption of DNA integrity (Agarwal et al., 2003b; Agarwal and Tamer, 2005a).

Recently, there has been increasing evidence suggesting that oxidative DNA damage is implicated in male fertility and sperm function (Agarwal et al., 2005a). Sperms like the other living aerobic cells are normally exposed to some reactive oxygen species (ROS) but if ROS level rise, oxidative stress occurs, which results in oxygen and

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Abbreviations: GPX, Glutathione peroxidase; **GRD,** glutathione reductase; **ROS,** reactive oxygen species; **TAC,** total antioxidant capacity; **FAD,** flavin adenine dinucleotide; **NADPH,** nicotinamide adenine dinucleotide phosphate; **NOS,** nitric oxide synthase; **nNOS,** neuronal NOS; **iNOS,** inducible NOS; **eNOS,** endothelial NOS.

oxygen-derived oxidants, and in turn increases the rates of cellular damage (Saleh and Agarwal, 2002). High concentrations of ROS cause lipid peroxidation, damage to cell membranes, proteins and DNA (Agarwal et al., 2005b).

There is a diverse range of antioxidants that limit the production of ROS, scavenge them and repair cell damage. Also, there is a complex interaction between the pro-oxidants and antioxidants that results in the maintenance of intracellular homeostasis. An imbalance between the pro-oxidants and antioxidants resulted in oxidative stress.

Nitric oxide (NO) is a free radical which is produced by most cells and tissues in the body and mediates a number of biological functions (Moncada et al., 1991). Nitric oxide is synthesized through the enzymatic conversion of L-arginine to L-citrulline by a family of isoenzymes known as the nitric oxide synthase (NOS) and involved in diverse physiological and pathophysiological processes in various organs, including the human male and female reproductive tracts (Forsterman et al., 1994; Rosselli et al., 1998). Three isoforms of NOS: NOS I, II and III, are known till date, as the alternate names for neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS), respectively (Forstermann et al., 1995).

It is well known that NO has a dual role on cells. Low concentrations of NO, are essential in biology and physiology of systems and plays an important antioxidant role that protects cells from oxidative stress (Moncada and Higgs, 1993; Hanafy et al., 2001; Hofmann et al., 2000), but excessive level of NO has a direct detrimental effect on cells, via the production of peroxynitrile, after interacting with oxygen or superoxide (Forstermann et al., 1995; Moncada and Higgs 1993; Davis et al., 2001). The ultimate effects of nitric oxide on cells depend on its interaction with hydrogen peroxide. Peroxyl nitrite anion may be formed *in vivo* from superoxide and nitric oxide and actively reacts with glutathione, cysteine, deoxyribose and other thiols/thioeters (Koppenol et al., 1992). With the identification of a unique isoform of NOS which is documented as testis specific nNOS (TnNOS) in the testis (Wang et al., 1997), an interest to examine the role of NOS and NO in spermatogenesis and steroidogenesis is expanded and a huge number of studies has been done to identify the role of NO in the male reproductive tract.

A large body of evidence derived from these studies which suggests that nitric oxide (NO) in low levels is involved in a variety of male reproductive processes such as spermatogenesis, spermiogenesis, sperm motion, sperm metabolism and sperm capacitation (Balercia et al., 2004; Romeo et al., 2003; Lee and Cheng, 2004; Lee and Cheng, 2008). However, high levels of nitric oxide have detrimental effects on sperm parameters. A negative correlation between nitric oxide levels in seminal plasma and sperm motility, morphology and DNA fragmentation have been reported (Balercia et al., 2004; Amiri et al., 2006; Huang et al., 2006; Amiri et al., 2007).

It is interesting to note that seminal plasma is well endowed with an array of antioxidant defense mechanisms to protect spermatozoa against oxidative stress (Armstrong et al., 1998). Seminal plasma contains a number of enzymatic antioxidants such as superoxide dismutase, the glutathione peroxidase/reductase (GPX/GRD) system and catalase. In addition, seminal plasma contains a variety of nonenzymatic antioxidants such as urate, ascorbate, pyruvate, glutathione, taurine and hypotaurin (Rasala and Agarwal, 2002). Studies have demonstrated that infertile men are more likely than fertile ones to have depressed total antioxidant capacity (TAC) and lower levels of individual antioxidants (Sharma et al., 1999).

The objectives of this study were: 1) To assess the level of nitric oxide in seminal plasma of fertile and infertile men, 2) to assess the status of the total antioxidant capacity in seminal plasma of fertile and infertile men, 3) to assess the sperm DNA damage in fertile and infertile men and 4) to assess the correlation of sperm DNA damage with nitric oxide concentration and total antioxidant capacity.

MATERIALS AND METHODS

A total of 115 semen samples were analyzed in this study, of these, 70 were obtained from semen donors with a normal semen analysis and 45 were provided by patients who had a history of infertility at least for 1 year and an abnormal semen analysis. No subjects in either group were smokers, on medication, had a history of exposure to chemotherapy or radiation or a varicocell. Semen samples were collected by masturbation after 3 - 5 days of sexual abstinence. Specimens were allowed to liquefy for 30 min at 37°C. After liquefaction, semen specimens were evaluated for sperm concentration and sperm motility according to the guidelines of the world health organization (1999). Morphology smears were scored using the Kruger's strict criteria (Kruger et al., 1998). Seminal leukocytes were quantified by a myeloperoxidase staining test and values were considered to be normal at concentration of $<1 \times 10^6$ peroxidase-positive leukocytes per milliliter of semen. Liquefied semen was centrifuged at 300 g for 10 min. The seminal plasma was aspirated and kept frozen until assayed for nitric oxide concentration and antioxidant capacity.

DNA damage assessment

Single cell gel electrophoresis (comet) assay

Spermatozoa were suspended in 1% (w/v) low melting point agarose (Sigma) at a concentration of 1×10^4 cell ml^{-1}. One milliliter of this suspension was applied to the surface of a microscope slide to form a microgel and allowed to set at 4°C for 5 min. Microgels were submersed in cell lysis buffer (comet assay 5 M NaCl, 100 mM EDTA, 10 mM tris HCl pH = 10, containing 1% triton X-100 and 40 mM dithiothritol) for 1 h at room temperature and protected from light. Following cell lysis, all slides were washed through three changes of deionized water at 20 min intervals to remove salt and detergent from the microgels. Slides were placed in a horizontal electrophoresis until they were allowed to equilibrate for 20 min with TBE buffer (10 mM Tris containing 0.08 M boric acid and 0.5 M EDTA pH = 8.2) before electrophoresis (25 V, 0.01A) for 20 min. When electrophoresis was complete, the slides were rinsed with water, air-dried and stored and protected from light analysis. After a

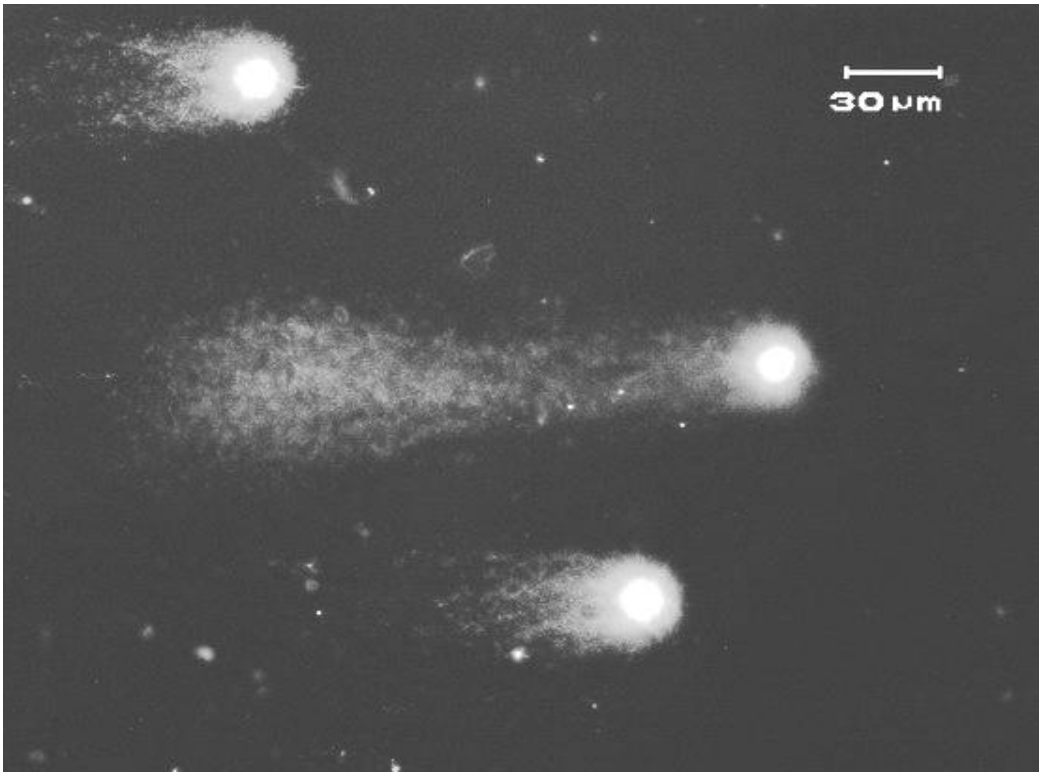

Figure 1. Comet formation in sperms.

neutralization step, the slides were each stained with propidium iodide (20 µg/ml) and mounted with a cover slip. Cells were visualized at 200x using a fluorescent microscope (Nikon). Each cell had the appearance of a comet (Figure 1) with a brightly florescent head and a tail to one side formed by the DNA, which contained strand breaks that were drown away during electrophoresis.

Samples were run in duplicate, and 50 cells were randomly analyzed per slide for a total of 100 cells per sample.

Nitrite and nitrate assay

Total nitrite and nitrate concentrations of seminal plasma were determined as measure of nitric oxide level. Nitrite was determined by a stepwise Griess reaction method. Nitrate was detected after reduction to nitrite using nitrate reductase. For nitrate reductase, the samples were centrifuged at 1000 g for 15 min at room temperature to remove cells and particles. Then, the 150 µl of samples incubated for 15 min at 30°C in the presence of 0.1 unit/ml nitrate reductase (from *Aspergillus*, Sigma), 5 µm FAD (Sigma), 30 mM NADPH (Sigma) in final volume of 160 µl. When nitrate reduction was completed, NADPH (up to 0.3 mM) was oxidized to avoid interference with the following nitrite determination. For this propose, the samples were incubated with 10 units/ml lactate dehydrogenase (from rabbit muscle, sigma) and 10 mM sodium pyruvate (sigma) for 5 min at 37°C in a final volume of 170 µl. Total nitrite was then determined spectrophotometrically by using Griess reaction by adding of 1% sulfanilamide and 0.1% naphthyl-ethlenediamine dihydrochloride in 2% phosphoric acid and by recording absorbance at 540 nm with a spectrophotometer after 10 min incubation at 37°C in the dark. Nitrite concentration was expressed as µmol/ml. Linear regression was used to determine NO concentration from standard carve of $NaNO_2$.

Total antioxidant capacity evaluation

In order to determine total antioxidant capacity, percentage of red blood cell (RBC) hemolysis in AAPH (2,2'-azobis comet assay-aminopropane dihydrochloride) medium was measured against different concentrations of ascorbic acid. 0.5 ml of seminal plasma with DNA ml of 10% suspension of RBC in PBS was mixed. Then 1 ml AAPH solution (100 mM) was added to the above solution and incubated at 37°C for 130 min. The tubes were shacked every 5 - 6 min during incubation. After incubation, tubes were centrifuged at 2000 rpm for 10 min. Absorbance of supernatant of the tubes was measured against phosphate saline buffer and subtracted from negative control tube. A negative control is a tube that contains DNA ml of 10% suspension of RBC and 1.5 ml of PBS (0.2 M) solution.

Statistical analysis

Data are reported as mean ± SD. The comparisons between two groups were made by t-test using SPSS-11. The linear dependence was measured using Pearson's correlation coefficient.

RESULTS

As shown in Table 1, the fertile group had significantly higher sperm concentration, motility and normal forms than the infertile group. The mean of DNA damage (comet percentage) in fertile men was 12.9 and in infertile men was 48.77 ($p < 0.001$).

Table 1. The mean of age, sperm parameters, NO level, TAC and comet index in normal and infertile groups.

Parameter	Normal	Infertile	P value
Sperm count (±SD)	63.78 (±)	39.64 (±)	P < 0.001
Sperm motility (±SD)	(±)	(±)	P < 0.001
Sperm morphology (±SD)	25.11(±6)	15.75 (±3.4)	P < 0.002
WBC count (±SD)	5.3 (±4.7)	15.75 (±10.3)	P < 0.002
NO concentration (±SD)	3.88 (±0.53)	5.47 (±1)	P < 0.001
TAC (±SD)	1243 (±575)	880 (±178)	P < 0.001
Comet index	12.9 (±17.5)	48.77 (±5.4)	P < 0.001

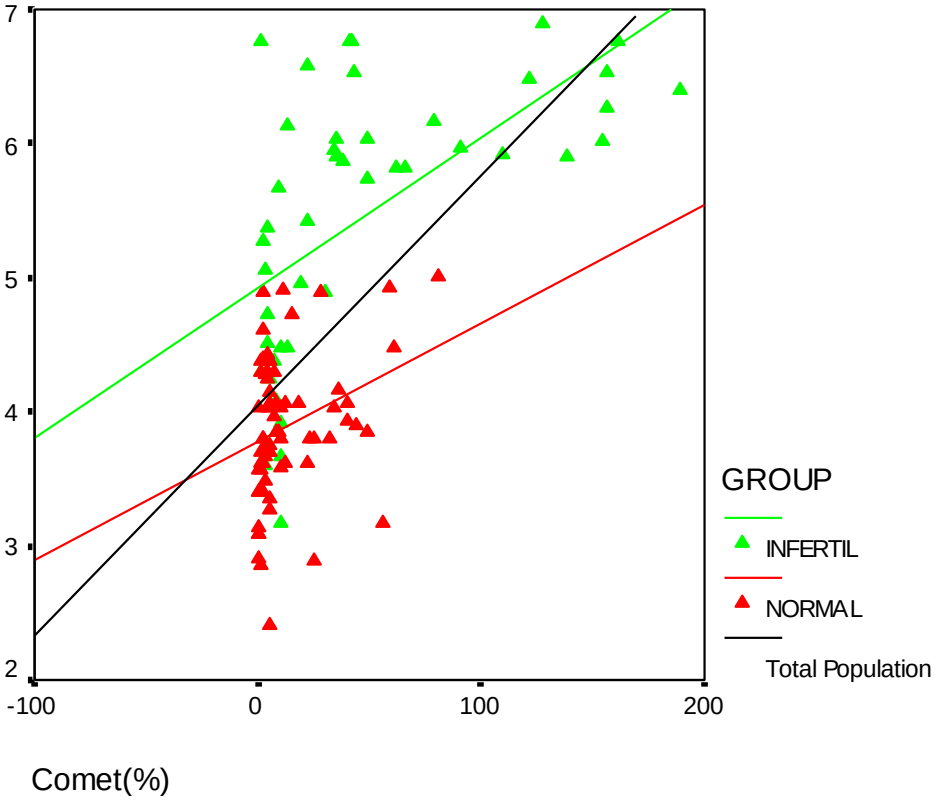

Figure 2. Positive linear correlations between nitric oxide (NO) concentration and comet percent in infertile and normal cases.

The mean of total nitrite level in the seminal plasma of infertile men was significantly higher than that of fertile men (5.47 ±1.1 versus 3.88 ± 0.53 µmol/l, p < 0.001). The means of TAC value in samples from normozospermic was 1243 ± 575, whereas in patients with abnormal semen parameters, it was 880 ± 178.

Analysis of total nitrite concentration, TAC and comet index using Pearson's correlation coefficient indicated that there were a significant positive correlation between nitrite levels with DNA damage in both group (Normal: p < 0.01, r = 0.294 and infertile: p < 0.001, r = 0.598) (Figure 2).

On the other hand, there was a significant negative correlation between DNA damage and TAC in infertile men (p < 0.04, r = - 0.3) (Figure 3). Also, a negative correlation was observed between nitric oxide concentration and TAC in infertile cases (p < 0.001, r = - 0.293) (Figure 4).

DISCUSSION

The results of this study show an important correlation between DNA damage with nitric oxide concentration and

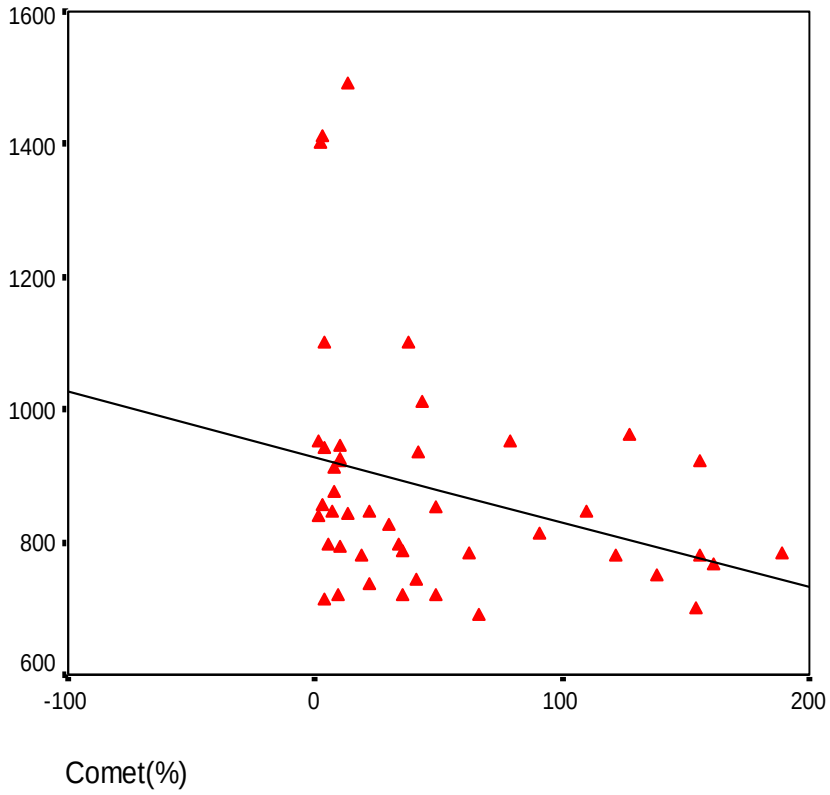

Figure 3. The figure shows negative linear correlations between total antioxidant capacity (TAC) and comet percent in infertile cases.

TAC in seminal plasma of infertile men. DNA and membrane phospholipids are the two main molecular targets for ROS. Oxidative DNA damage refers to various types of functional or structural changes due to the reaction of ROS with DNA. Sperm DNA is believed to be especially vulnerable to oxidative stress, partially owing to: 1) the high content of polyunsaturated fatty acids in their cell membranes and 2) the relative weakness of the antioxidant system in seminal fluid (Shen et al., 1999).

The primary mechanism of nitric oxide-induced sperm damage is likely to be inhibition of mitochondrial respiration and DNA synthesis (Kobayashi et al., 1991).

High concentrations of nitric oxide have a deleterious effect on spermatozoa kinetic characteristics. As a possible explanation, it is speculated that nitric oxide may react with superoxide or hydrogen peroxide, resulting in the formation of peroxynitrite hydroxyl radical and singlet oxygen, which causes oxidation of sperm membrane, lipid and thiol proteins. Herreo et al. (1997) have reported that nitric oxide is capable of regulating cyclic adenosine monophosphoate (cAMP) concentration and consequently, capacitation could act directly by targeting the enzyme or by altering the action of a distinct regulatory protein. The sensitivity of sulfhydryl groups of proteins to both nitrosative and oxidative events which in turn may elicit distinct functional changes has been speculated.

Morphologically abnormal spermatozoa and seminal leukocytes have been established as the main sources of high ROS production in human ejaculated sperm (Kessopoulou et al., 1992). Activated leukocytes are capable of producing 100-fold higher amounts of ROS than no activated leukocyte (Plante, 1994). Leukocytes may be activated in response to a variety of stimuli including inflammation and infection (Pasqualotto et al., 2000). Total antioxidant capacity of seminal plasma receives an important contribution from the epididymis which possesses region–specific antioxidant activity. It may potentially protect spermatozoa from oxidative attack during storage at this site (Mancini et al., 2004).

Potts et al. (1999) reported that seminal plasma from men who have had vasectomies contains less total antioxidant capacity, lower thiol group concentrations and higher amounts of lipid peroxidation compared with men with an intact ductile system.

In conclusion, the main findings of this study are that the sperm DNA damaged and nitric oxide concentration in semen samples of infertile men are higher than those of normozoospermic fertile men, and TAC in infertile men is lower than fertile men. We found that there was a positive correlation between TAC with DNA damage and nitric oxide levels in both group. The present data suggest that the over production of free radicals and the consequent

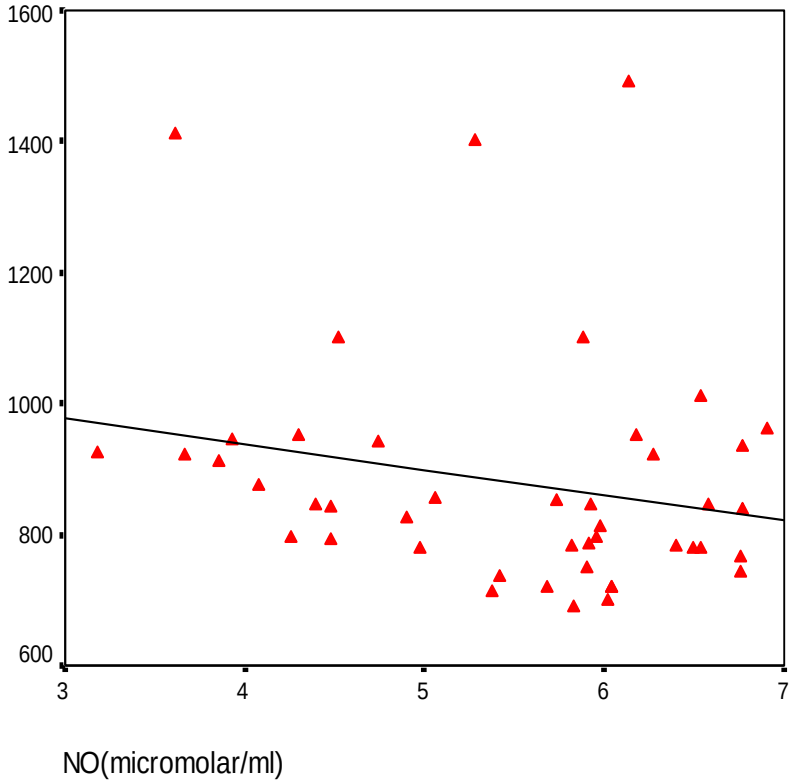

Figure 4. Negative linear correlation between total antioxidant capacity (TAC) and NO concentration in infertile cases.

excessive exposure to oxidative conditions have a potential pathogenetic role in sperm DNA damage and male infertility.

ACKNOWLEDGEMENTS

This study was supported by Hamadan University of Medical Sciences and was done in Fatemieh Infertility Center and Research Center for Molecular Medicine. The authors of this article thank the directors.

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