# Lipid Peroxidation and Nitric Oxide Levels in Male Smokers' Spermatozoa and their Relation with Sperm Motility

Mohammad Ali Ghaffari<sup>1,2,3\*</sup>, Morad Rostami<sup>3</sup>

1- Physiology Research Center, Ahwaz Jundishapur University of Medical Sciences, Ahwaz, Iran

2- Cellular and Molecular Research Center, Ahwaz Jundishapur University of Medical Sciences, Ahwaz, Iran

3- Department of Biochemistry, School of Medicine, Ahwaz Jundishapur University of Medical Sciences, Ahwaz, Iran

#### Abstract

**Background:** Nitric oxide (NO) is synthesized from L-arginine by a family of enzymes known as nitric oxide synthases. Low concentrations of NO is essential in biology and physiology of spermatozoa, but high amounts of NO is toxic and has negative effects on sperm functions. Moreover, sperm membrane contains high concentrations of polyunsaturated fatty acids that are highly susceptible to oxidative damage that interferes with fertilization ability. Therefore, we investigated the correlation between levels of sperm malondialdehyde (MDA) and NO with sperm motility in male smokers.

**Methods:** Semen samples were collected from normozoospermic smoker (n=64) and nonsmoker (n=83) men. The content of sperm lipid peroxidation was determined by measuring malondialdehyde (MDA). The sperm NO were also measured using Griess reagent. Data was analyzed by SPSS, (version 15.0), using independent t-test and Pearson analysis.

**Results:** The mean MDA and NO concentrations in the sperm of normozoospermic male smokers were significantly higher than the control group or normozoospermic nonsmokers, (p < 0.001). A significant negative relationship was noted between sperm motility and sperm MDA levels (r=-0.32, p=0.01); and sperm motility and sperm NO concentration (for nitrite, r=-0.34, p=0.006 and for nitrate, r=-0.38, p= 0.002).

**Conclusion:** It was concluded that the increase in MDA and NO production in sperm can influence sperm motility in normozoospermic smokers. Therefore, it seems that cigarette smoking may affect the fertility of male smokers via increasing the amount of sperm MDA/lipid peroxidation and NO concentrations.

**Keywords:** Cigarette smoking, Human sperm, Lipid peroxidation, Nitric oxide, Smoker men. **To cite this article:** Ghaffari MA, Rostami M. Lipid Peroxidation and Nitric Oxide Levels in Male Smokers' Spermatozoa and their Relation with Sperm Motility. J Reprod Infertil. 2012;13(2):81-87.

#### Introduction

n mammalian germ cells, reactive oxygen species (ROS) production, including hydrogen peroxide ( $H_2O_2$ ), superoxide anion ( $O_2^-$ ), and/or hydroxyl radical ( $\cdot$ OH), has been shown to be a physiological event required for maturation, capacitation, acrosomal reaction of spermatozoa, binding with the zona pellucida, and oocyte fusion (1). However, excessive generation of ROS damages the structural and functional parameters of sperm (2). Antioxidants, including superoxide dismutase, glutathione peroxidase, and catalase play an important defensive role in neutralizing ROS (3). The balance between ROS generation and antioxidants should be finely tuned in order to protect sperm (3). All cellular components, including polyunsaturated fatty acids, proteins, and nucleic acids, are potential targets of ROS. However, sperm's membrane polyunsaturated fatty

\* Corresponding Author: Mohammad Ali Ghaffari, Department of Biochemistry, School of Medicine, Ahwaz Jundishapur University of Medical Sciences, Ahwaz, Iran *E-mail:* ghaffari@ajums.ac.ir ghaffarima@yahoo.com

**Received:** Nov. 5, 2011 **Accepted:** Jan. 28, 2012

J Reprod Infertil. 2012;13(2):81-87

## **JRI** Smoking and Male Fertility

acids have a critical role in sperm function because lipid peroxidation leads to a loss of integrity in the sperm plasma membrane (4).

A lot of studies support the role of nitric oxide as a messenger in a wide array of biological processes such as neurotransmission (5), regulation of vascular wall tone (6), and immune system activity as an effector molecule with bactericidal and antiviral properties (7). NO also modulates sexual and reproductive functions in mammalian species (8). NO is a free radical which is generated from the oxidation of L-arginin to L-citrulline by nitric oxide synthase (NOS) isozymes (9). NOS activity has been detected in human and rat testis, epididymis, prostate, and seminal vescicles (10). Several studies have shown that this enzyme is associated with acrosome and tails of mouse and human sperm and it appears to be involved in sperm motility and acrosomal reaction that are the main factors in fertilization process (11-13).

Several studies have demonstrated that certain chemicals present in cigarette smoke can adversely affect male fertility (14, 15). Some of these compounds, especially nicotine and its metabolite cotenine, and cadmium have been found in seminal plasma at a concentration proportional to those in serum; therefore, these substances seem to be able to pass from blood-testis barrier (16). Consequently, seminal plasma of male smokers can be considered as a toxic environment for sperm. A relationship has also been found between cigarette smoking and alteration of sperm quality (14, 17, 18). Indeed, cigarette smoking is linked to increased levels of ROS in seminal plasma (15).

Although cigarette smoking is a hazardous habit but more people seem to consume cigarettes on a regular basis. The highest prevalence of smoking is observed in young males, aged 20–39 years, during their reproductive age (19).

Although a lot of studies have been done on the effects of smoking on male reproductive function (14, 15, 19, 21), but the role of cigarette smoking on nitric oxide and lipid peroxidation levels in sperm seem to be limited in the literature. Therefore, the objective of the present study was to evaluate the effects of cigarette smoking on the amount of sperm nitric oxide and sperm oxidation by measuring the levels of nitrite (NO<sub>2</sub><sup>-</sup>), nitrate (NO<sub>3</sub><sup>-</sup>), and malondialdehyde (MDA) in the sperm of normozoospermic men with the habit.

### Methods

The study was approved by the institutional review board of the Institute Research Committee of Ahwaz Jundishapour University of Medical Sciences.

Semen samples were obtained from male partner of couples attending Razi Laboratory in Ahwaz, Iran for a routine semen analysis. Subsequently, a questionnaire was distributed to obtain demographic data, smoking habits, alcohol use, and use of other substances and drugs. Individuals who had consumed alcohol and/or narcotic drugs in the past three months were not eligible for the study. Other exclusion criteria included recent fever or exposure to gonadotoxins (*e.g.* chemotherapy or radiotherapy), to pesticides or heavy metals (professionally). None of the participants were receiving or had received any kind of vitamin treatment in the past three months.

A total of 147 men with a mean age of 30 years (ranging from 17-41 years) provided 147 semen sample for analysis. The 147 men categorized according to the number of cigarettes they usually smoked per day over three months before providing the sample, were divided into two groups of nonsmokers (n=83) and smokers (n=64).

Semen samples were collected by masturbation into a sterile container after 2-3 days of sexual abstinence. Following a ten-minute liquefaction period of semen at 37 °C and 5% CO<sub>2</sub>, samples were examined for volume, sperm motility and count according to World Health Organization guidelines (22). All the collected samples had a volume of  $\geq 3.0 \ ml$  and sperm concentration/ mLof  $\geq 20 \times 10^6$ . Sperm were separated from seminal plasma by centrifugation at 1000 g for 10 minutes at room temperature. The sperm were washed twice by adding phosphate buffer (20 mM, pH= 7.4) and centrifuged at 2500 g for 5 minutes. Finally, sperm samples were resolved in phosphate buffer and were aliquoted as a homogenous mixture with  $1 \times 10^6$  sperm. The aliquots were stored at -80 °C for nitrite, nitrate, and malondialdehyde assays.

*Sperm lipid peroxidation determination:* Lipid peroxides, derived from polyunsaturated fatty acids, are unstable molecules which decompose to form a complex series of compounds, most abundantly malondialdehyde (MDA) (23). Therefore, content of sperm lipid peroxidation was determined by measuring MDA as described by Rao et al. (23). Sperm samples were lysed using rapid freeze and

Results

thaw method, respectively at -80 °C and 35 °C, for least three times. The samples were centrifuged at 4000 g for 10 minutes, and the supernatant was used for the assays. The MDA level of each sample was measured by its color reaction with thiobarbituric acid reagent at 100 °C for one hour, which its maximum absorbance is at 534 nm (23). The concentration of MDA was calculated using the extinction coefficient of  $1.56 \times 10^5 \text{ mol.1}^{-1}.\text{cm}^{-1}$ and expressed as nmol of MDA equivalents per  $10^6$  sperm.

Sperm NO analysis: NO assay is difficult because it decomposes rapidly into nitrite  $(NO_2)$  and nitrate  $(NO_3)$  in biological solutions. Therefore,  $NO_2$  and  $NO_3$  assay is often used as a measure of NO radical production. Sperm specimens lysed through rapid freeze-thawing at -80  $^{\circ}C$  and 35  $^{\circ}C$ , for three times respectively. The sperm NO<sub>2</sub><sup>-</sup> levels were measured using the Griess reagent, as previously described (24, 25). The Griess reagent consists of sulfanilamide (58 mM in 3 M HCl) and N-1-naphtylethylenediamine (722  $\mu M$ ). It is necessary to use protein-free samples for NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup> assays; therefore, Somogyi method is used to eliminate protein interference (26). Briefly, 8 ml distilled water, 0.5 ml zinc sulphate (10%), and 0.5 ml NaOH (0.5 N), were added to 1 ml of semen sample. The mixture was later centrifuged for 10 minutes at 4000 g, and finally the supernatant was collected. NO<sub>2</sub><sup>-</sup> concentration of sperm samples was determined by spectrophotometry at 540 nm with the addition of Griess reagent to deproteinized samples converting NO<sub>2</sub><sup>-</sup> into a deep purple azo compound. In this study,  $0.78-50 \ \mu M$ concentration of sodium nitrite was used for plotting the standard curve, and the results were reported as  $nmol/10^6$  sperm.

 $NO_3^-$  measurement is based on a two-step procedure. The first step is the reduction of  $NO_3^-$  to  $NO_2^-$  using copper coated cadmium granules, as previously reported (25). The second step is the addition of Griess reagent, as described in above.

Statistical analysis: All assays were performed in triplicate and the mean $\pm$ SD was used for the calculation. T-test was employed for comparisons between sperm nitrite, nitrate, and malondialdehyde levels in smoker and nonsmoker men. The coefficients of correlation were calculated analyzed by linear (Pearson) analysis. According to "one sample kolmogorov-smirnov test" data distribution are normal. Significance was defined as p  $\leq 0.05$ .

The series studied included a total of 147 normozoospermic men who were, divided into two groups of smokers (n=64) and nonsmokers (n= 83). Smokers had consumed 7 to 40 cigarettes per day (16±7.5 cigarettes/day) for a duration of 1 to 20 years (6±4 years). Comparing sperm motility, lipid peroxidation levels (using MDA assay), and NO levels (in forms of nitrite and nitrate) between the two groups showed a significant decrease in motility (p=0.004) and a significant increase in concentrations of MDA, nitrite, and nitrate (p <0.001) in sperm of smokers (Table 1). Correlations between sperm motility and levels of MDA, nitrite, and nitrate in sperm of smokers have been shown in Figure 1. This figure, shows a significantly negative correlation between sperm motility and concentrations of MDA (r=-0.32; p=0.01), nitrite (r=-0.34; p=0.006), and nitrate (r=-0.38; p=0.002) in sperm of male smoker. A significant positive relation was found between MDA levels in sperm of the smoker group with the number of cigarettes smoked per day (r=0.48; p <0.001) and/ or duration of cigarette smoking per year (r=0.66; p < 0.001) (Figure 2). However, the relationship was not statistically significant between nitrite and nitrate content of sperm cells with the number of consumed cigarettes per day (r=0.07; p=0.5 and r= -0.02; p=0.8, respectively) and/or cigarette smoking duration of per year (r=-0.04; p=0.7 and r= -0.01; p=0.9, respectively) (Data not shown). Finally, a positive but not significant relation was observed between concentration of MDA in sperm with both nitrite (r=0.20; p=0.1) and nitrate levels (r=0.19; p=0.1) in sperm of smoker men (Figure 3).

 Table 1. Semen analysis and comparison of motility and concentration of MDA, nitrite, and nitrate in the sperm of normo-zoospermic smokers and non-smokers

zoosperinie sinokers and non-sinokers		
Characteristics	Non-smoker (n=83)	Smoker (n=64)
Age (year)	29.27±4.96	29.70±4.45 <sup>a</sup>
Volume ( <i>ml</i> )	$4.10 \pm 1.40$	3.20±1.06 <sup>b</sup>
рН	8.01±0.22	$8.03 \pm 0.32$ °
Sperm concentration (10 <sup>6</sup> /ml)	65.50±22.90	52.80±20.70 <sup>b</sup>
Sperm morphology (%)	47.50±18.90	37.00±21.20 <sup>b</sup>
Sperm motility (%)	46.50±13.80	42.50±20.20 <sup>b</sup>
Sperm MDA ( <i>nmol/</i> 10 <sup>6</sup> sperm)	$0.14{\pm}0.05$	0.27±0.10 <sup>b</sup>
Sperm NO <sub>2</sub> <sup>-</sup> ( <i>nmol</i> /10 <sup>6</sup> sperm)	0.41±0.12	0.77±0.25 <sup>b</sup>
Sperm NO <sub>3</sub> <sup>-</sup> ( <i>nmol</i> /10 <sup>6</sup> sperm)	$0.78 \pm 0.22$	1.36±0.46 <sup>b</sup>

MDA: malondialdehyde concentration; NO<sub>2</sub>: nitrite concentration; No<sub>3</sub>: nitrate concentration. a: p=0.5, b: p  $\le$ 0.01, c: p=0.66

JRI Smoking and Male Fertility

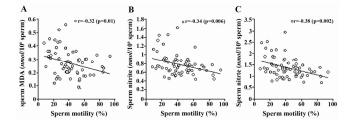
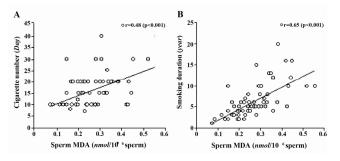


Figure 1. The relationship between sperm motility and concentration of sperm MDA, A) nitrite, B) and nitrate (C) in male smokers



**Figure 2.** The relationship between sperm MDA concentration and the number of cigarette, A) and duration of smoking, B) in male smokers

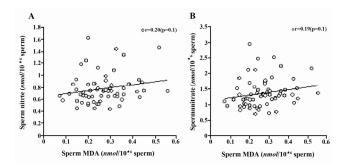


Figure 3. The relationship between sperm MDA and sperm nitrite, A) and nitrate concentrations, B) in male smokers

#### Discussion

Cigarette smoke contains several oxidant chemicals (15, 44), which could have an important role in lipid peroxidation processes within sperm.

In the present study, we observed a significantly decreased sperm motility (by 9%) and a significantly increased MDA, nitrite, and nitrate concentrations (by 48%, 47%, and 43%, respectively), in the sperm of normozoospermic smokers in comparison with non-smoker men. Several reports support the negative effect of cigarette smoke on sperm motility; for instance, Calogero et al. (27) demonstrated cigarette smoke extract suppressing sperm motility in a concentration- and time- dependent manner. Similarly, Hung et al. (28, 29) also reported that in vitro and/or in vivo tobacco

smoke treatment decrease the percentage of motile sperm and motility parameters in adult rhesus monkeys. However, the literature concerning the effects of cigarette smoking on sperm MDA, nitrite, and nitrate concentration in fertile men on smoking is limited. Non the less, There were some studies on levels of these substances in semen and/or other parts of the reproductive system of smoker and/or non-smoker infertile men (30–33).

Numerous studies have shown oxidative stress to play a key role in the pathophysiology of sperm in human (4, 34, 35). Hsieh et al. (36) obtained a negative correlation between MDA concentration and motility of sperm in oligoasthenospermic men. They suggested that increased MDA levels could inhibit sperm motility by pathologically affecting sperm membrane. In contrast, some other researchers have reported that MDA levels in the seminal plasma were not correlated with motility or concentration of sperm (37, 38). Similarly, Aleksandra et al. (39) did not obtain any correlation between MDA concentration and sperm parameters.

Several reports have indicated that these deleterious reactive compounds are usually suppressed by the coordinated functioning of seminal enzymatic and nonenzymatic antioxidants. For instance, Geva et al. (3) demonstrated that use of antioxidants led to a reduction in MDA, which was correlated with improvement in the prevalence of fertilization. Other reports indicate that enzymatic antioxidants, such as glutathione peroxidase (40), and SOD (41), as well as nonenzymatic antioxidants, such as vitamin E (42), and vitamin C (43), could increase sperm motility by reducing MDA activity. In the present study, evaluation of the MDA content of sperm had a significant (p=0.01) negative correlation with sperm motility in normozoospermic smokers. In addition, we observed a positive and significant relationship between both the number of cigarette smoked per day (p <0.001) and duration of cigarette smoking per year (p <0.001) with concentration of MDA in sperm. These results suggest that cigarette smoke could increase sperm MDA levels in a concentration- and time-dependent manner.

Nitric oxide (NO) is a large molecule which plays an important role in sperm physiology (45). Lewis et al. (46) showed that sperm were sources of NO and constitutive nitric oxide synthase (NOS) which is present in two isoforms similar to those present in both endothelial (ecNOS) and brain (bNOS) cells. NO itself is a highly reactive, short-lived, and lipophilic molecule with a halflife of just a few seconds which makes it difficult to measure (47). Therefore, its metabolites, nitrite and nitrate, were studied in the present study. Our study indicated a significant negative correlation between sperm motility and levels of both nitrite (p=0.006) and nitrate (p=0.002) in sperm of normozoospermic smokers. However, no significant correlation was found between sperm nitrite and/ or nitrate concentration with the number and/or duration of cigarette smoking. Similarly, except one report (48) that demonstrated sperm motility could not be affected by NO levels, other investigations have shown decreased sperm motility in the presence of high concentrations of NO-releasing compounds, such as sodium nitroprussie (49–51). Thus, result of this study being in agreement with other reports, we suggest that cigarette smoking can increase NO concentration in sperm in a concentration- and time- independent manner.

Weinberg et al. (51) demonstrated that NO could reduce ATP levels in cells via a decrease in ATP content and/or production as approximately 90% of the energy in sperm is produced as ATP (51); therefore, this mechanism may probably be responsible for a significant negative correlation observed between sperm nitrite and nitrate levels with sperm motility, in this study.

Finally, this investigation indicated a positive but insignificant correlation between MDA content of sperm with nitrite and nitrate levels in male smokers. This suggests that the elevated lipid peroxidation in male smokers' sperm has not solely occured by NO production. Therefore, cigarette smoking probably could increase MAD/lipid peroxidation in sperm via production of NO and other oxidative agents. In the present study, we excluded samples presenting leukocytospermia, thereby, allowing us to demonstrate these molecular alterations had been caused by tobacco and not mediated by leucocytes. In support of our hypothesis, there are a lot of reports that demonstrate production of different oxidative agents, such as superoxide anion  $(O_2^{-})$ , hydrogen peroxide  $(H_2O_2)$ , proxyl radical (ROO<sup>-</sup>) and hydroxyl radical (OH<sup>-</sup>) in the male reproductive system (52-54).

### Conclusion

In conclusion, the present study indicate an association between cigarette smoking and an increase in MDA/lipid peroxidation levels duo to an increase in the concentration of NO and probably other oxidative factors in the sperm of normozoospermic cigarette smokers. In addition, it was also demonstrated that high levels of MDA and NO have a significant negative correlation with sperm motility. Therefore, we suggest cigarette components may potentially affect motility of sperm via increase in MDA/lipid peroxidation, and NO concentrations in sperm. Thus, high levels of sperm MDA and NO may be one of the possible factors of fertility in male smokers. However, further investigations are needed to elucidate the exact effect of cigarette smoking on sperm activity in male smokers.

#### Acknowledgement

This work was financially supported by a grant for Project No. PRC-47 from Physiology Research Center of Ahwaz Jundishapur University of Medical Sciences in Ahwaz, Iran.

#### References

- de Lamirande E, Gagnon C. Human sperm hyperactivation and capacitation as parts of an oxidative process. Free Radic Biol Med. 1993;14(2):157-66.
- 2. Henkel R. The impact of oxidants on sperm function. Andrologia. 2005;37(6):205-6.
- 3. Geva E, Bartoov B, Zabludovsky N, Lessing JB, Lerner-Geva L, Amit A. The effect of antioxidant treatment on human spermatozoa and fertilization rate in an in vitro fertilization program. Fertil Steril. 1996;66(3):430-4.
- 4. Agarwal A, Gupta S, Sikka S. The role of free radicals and antioxidants in reproduction. Curr Opin Obstet Gynecol. 2006;18(3):325-32.
- Peunova N, Enikolopov G. Amplification of calcium induced gene transcription by nitric oxide in neuronal cells. Nature. 1993;364(6436):450-3.
- Calver A, Collier J, Vallance P. Nitric oxide and cardiovascular control. Exp Physiol. 1993;78(3): 303-26.
- Karupiah G, Xie QW, Buller RM, Nathan C, Duarte C, MacMicking JD. Inhibition of viral replication by interferon-gamma-induced nitric oxide synthase. Science. 1993;261(5127):1445-8.
- Zini A, O'Bryan MK, Magid MS, Schlegel PN. Immunohistochemical localization of endothelial nitric oxide synthase in human testis, epididymis, and vas deferens suggests a possible role for nitric oxide in spermatogenesis, sperm maturation, and programmed cell death. Biol Reprod. 1996;55(5):935-41.
- 9. Förstermann U, Closs EI, Pollock JS, Nakane M, Schwarz P, Gath I, et al. Nitric oxide synthase isozymes. Characterization, purification, molecular

## **JRI** Smoking and Male Fertility

cloning, and functions. Hypertension. 1994;23(6 Pt 2):1121-31.

- Burnett AL, Ricker DD, Chamness SL, Maguire MP, Crone JK, Bredt DS, et al. Localization of nitric oxide synthase in the reproductive organs of the male rat. Biol Reprod. 1995;52(1):1-7.
- Herrero MB, Pérez Martínez S, Viggiano JM, Polak JM, de Gimeno MF. Localization by indirect immunofluorescence of nitric oxide synthase in mouse and human spermatozoa. Reprod Fertil Dev. 1996;8(5):931-4.
- Herrero MB, Viggiano JM, Pérez Martínez S, de Gimeno MF. Evidence that nitric oxide synthase is involved in progesterone-induced acrosomal exocytosis in mouse spermatozoa. Reprod Fertil Dev. 1997;9(4):433-9.
- Belén Herrero M, Chatterjee S, Lefièvre L, de Lamirande E, Gagnon C. Nitric oxide interacts with the cAMP pathway to modulate capacitation of human spermatozoa. Free Radic Biol Med. 2000; 29(6):522-36.
- Zenzes MT. Smoking and reproduction: gene damage to human gametes and embryos. Hum Reprod Update. 2000;6(2):122-31.
- Sepaniak S, Forges T, Gerard H, Foliguet B, Bene MC, Monnier-Barbarino P. The influence of cigarette smoking on human sperm quality and DNA fragmentation. Toxicology. 2006;223(1-2):54-60.
- Pacifici R, Altieri I, Gandini L, Lenzi A, Pichini S, Rosa M, et al. Nicotine, cotinine, and trans-3-hydroxycotinine levels in seminal plasma of smokers: effects on sperm parameters. Ther Drug Monit. 1993;15(5):358-63.
- 17. Vine MF. Smoking and male reproduction: a review. Int J Androl. 1996;19(6):323-37.
- Sofikitis N, Takenaka M, Kanakas N, Papadopoulos H, Yamamoto Y, Drakakis P, et al. Effects of cotinine on sperm motility, membrane function, and fertilizing capacity in vitro. Urol Res. 2000;28 (6):370-5.
- Künzle R, Mueller MD, Hänggi W, Birkhäuser MH, Drescher H, Bersinger NA. Semen quality of male smokers and nonsmokers in infertile couples. Fertil Steril. 2003;79(2):287-91.
- Zhang JP, Meng QY, Wang Q, Zhang LJ, Mao YL, Sun ZX. Effect of smoking on semen quality of infertile men in Shandong, China. Asian J Androl. 2000;2(2):143-6.
- Benowitz NL, Jacob P 3rd, Herrera B. Nicotine intake and dose response when smoking reducednicotine content cigarettes. Clin Pharmacol Ther. 2006;80(6):703-14.

- 22. World Health Organization. [Laboratory manual of the WHO for the examination of human semen and sperm-cervical mucus interaction]. Ann Ist Super Sanita. 2001;37(1):I-XII, 1-123. Italian.
- Rao B, Soufir JC, Martin M, David G. Lipid peroxidation in human spermatozoa as related to midpiece abnormalities and motility. Gamete Res. 1989;24(2):127-34.
- 24. Moshage H, Kok B, Huizenga JR, Jansen PL. Nitrite and nitrate determinations in plasma: a critical evaluation. Clin Chem. 1995;41(6 Pt 1):892-6.
- Ghaffari MA, Kadkhodaei-Elyaderani M, Saffari MR, Pedram M. Monitoring of serum nitric oxide in patients with acute leukemia. Iran J Pharm Res. 2005;4(4):233-7.
- Somogyi M. A method for the preparation of blood filtrates for the determination of sugar. J Biol Chem. 1930;86:655-63.
- Calogero A, Polosa R, Perdichizzi A, Guarino F, La Vignera S, Scarfía A, et al. Cigarette smoke extract immobilizes human spermatozoa and induces sperm apoptosis. Reprod Biomed Online. 2009;19 (4):564-71.
- Hung PH, Baumber J, Meyers SA, VandeVoort C A. Effects of environmental tobacco smoke in vitro on rhesus monkey sperm function. Reprod Toxicol. 2007;23(4):499-506.
- Hung PH, Froenicke L, Lin CY, Lyons LA, Miller MG, Pinkerton KE, et al. Effects of environmental tobacco smoke in vivo on rhesus monkey semen quality, sperm function, and sperm metabolism. Reprod Toxicol. 2009;27(2):140-8.
- Romeo C, Ientile R, Santoro G, Impellizzeri P, Turiaco N, Impalà P, et al. Nitric oxide production is increased in the spermatic veins of adolescents with left idiophatic varicocele. J Pediatr Surg. 2001;36(2):389-93.
- Türkyilmaz Z, Gülen S, Sönmez K, Karabulut R, Dinçer S, Can Başaklar A, et al. Increased nitric oxide is accompanied by lipid oxidation in adolescent varicocele. Int J Androl. 2004;27(3):183-7.
- 32. Başar MM, Kisa U, Tuğlu D, Yilmaz E, Başar H, Cağlayan O, et al. Testicular nitric oxide and thiobarbituric acid reactive substances levels in obstructive azoospermia: a possible role in pathophysiology of infertility. Mediators Inflamm. 2006; 2006(3):27458.
- Battaglia C, Giulini S, Regnani G, Madgar I, Facchinetti F, Volpe A. Intratesticular Doppler flow, seminal plasma nitrites/nitrates, and nonobstructive sperm extraction from patients with obstructive and nonobstructive azoospermia. Fertil Steril. 2001;75 (6):1088-94.

- 34. Cocuzza M, Sikka SC, Athayde KS, Agarwal A. Clinical relevance of oxidative stress and sperm chromatin damage in male infertility: an evidence based analysis. Int Braz J Urol. 2007;33(5):603-21.
- 35. Garrido N, Meseguer M, Simon C, Pellicer A, Remohi J. Pro-oxidative and anti-oxidative imbalance in human semen and its relation with male fertility. Asian J Androl. 2004;6(1):59-65.
- Hsieh YY, Chang CC, Lin CS. Seminal malondialdehyde concentration but not glutathione peroxidase activity is negatively correlated with seminal concentration and motility. Int J Biol Sci. 2006;2(1):23-9.
- 37. Kobayashi T, Miyazaki T, Natori M, Nozawa S. Protective role of superoxide dismutase in human sperm motility: superoxide dismutase activity and lipid peroxide in human seminal plasma and spermatozoa. Hum Reprod. 1991;6(7):987-91.
- Suleiman SA, Ali ME, Zaki ZM, el-Malik EM, Nasr MA. Lipid peroxidation and human sperm motility: protective role of vitamin E. J Androl. 1996;17(5):530-7.
- Aleksandra K, Slawomir K, Ewa B. Values of malondialdehyde in human seminal plasma. Prog Med Res. 2004;2:1-10.
- Alvarez JG, Storey BT. Role of glutathione peroxidase in protecting mammalian spermatozoa from loss of motility caused by spontaneous lipid peroxidation. Gamete Res. 1989;23(1):77-90.
- Alvarez JG, Storey BT. Evidence for increased lipid peroxidative damage and loss of superoxide dismutase activity as a mode of sublethal cryodamage to human sperm during cryopreservation. J Androl. 1992;13(3):232-41.
- 42. Verma A, Kanwar KC. Effect of vitamin E on human sperm motility and lipid peroxidation in vitro. Asian J Androl. 1999;1(3):151-4.
- 43. Fraga CG, Motchnik PA, Shigenaga MK, Helbock HJ, Jacob RA, Ames BN. Ascorbic acid protects against endogenous oxidative DNA damage in human sperm. Proc Natl Acad Sci U S A. 1991;88 (24):11003-6.
- Hammond D, Fong GT, Cummings KM, O'Connor RJ, Giovino GA, McNeill A. Cigarette yields and human exposure: a comparison of alternative testing regimens. Cancer Epidemiol Biomarkers Prev. 2006;15(8):1495-501.

- 45. Aitken J, Fisher H. Reactive oxygen species generation and human spermatozoa: the balance of benefit and risk. Bioessays. 1994;16(4):259-67.
- 46. Lewis SE, Donnelly ET, Sterling ES, Kennedy M S, Thompson W, Chakravarthy U. Nitric oxide synthase and nitrite production in human spermatozoa: evidence that endogenous nitric oxide is beneficial to sperm motility. Mol Hum Reprod. 1996;2 (11):873-8.
- 47. Ignarro LJ, Gold ME, Buga GM, Byrns RE, Wood KS, Chaudhuri G, et al. Basic polyamino acids rich in arginine, lysine, or ornithine cause both enhancement of and refractoriness to formation of endothelium-derived nitric oxide in pulmonary artery and vein. Circ Res. 1989;64(2):315-29.
- Tomlinson MJ, East SJ, Barratt CL, Bolton AE, Cooke ID. Preliminary communication: possible role of reactive nitrogen intermediates in leucocyte-mediated sperm dysfunction. Am J Reprod Immunol. 1992;27(1-2):89-92.
- Herrero MB, Cebral E, Boquet M, Viggiano JM, Vitullo A, Gimeno MA. Effect of nitric oxide on mouse sperm hyperactivation. Acta Physiol Pharmacol Ther Latinoam. 1994;44(3):65-9.
- Rosselli M, Dubey RK, Imthurn B, Macas E, Keller PJ. Effects of nitric oxide on human spermatozoa: evidence that nitric oxide decreases sperm motility and induces sperm toxicity. Hum Reprod. 1995;10(7):1786-90.
- 51. Weinberg JB, Doty E, Bonaventura J, Haney AF. Nitric oxide inhibition of human sperm motility. Fertil Steril. 1995;64(2):408-13.
- Maneesh M, Jayalekshmi H. Role of reactive oxygen species and antioxidants on pathophysiology of male reproduction. Indian J Clin Biochem. 2006;21 (2):80-9.
- 53. Baumber J, Ball BA, Gravance CG, Medina V, Davies-Morel MC. The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential, and membrane lipid peroxidation. J Androl. 2000; 21(6):895-902.
- 54. Cocuzza M, Sikka SC, Athayde KS, Agarwal A. Clinical relevance of oxidative stress and sperm chromatin damage in male infertility: an evidence based analysis. Int Braz J Urol. 2007;33(5):603-21.