Effect of cigarette smoking on antioxidant levels and presence of leukocytospermia in infertile men: a prospective study

Fábio Firmbach Pasqualotto, M.D., Ph.D., Fernanda Medeiros Umezu, Ms.C., Mirian Salvador, Ph.D., Edson Borges, Jr, M.D., Ph.D., Bernardo Passos Sobreiro, M.D., Ph.D., and Eleonora Bedin Pasqualotto, M.D., Ph.D.

Instituto de Biotecnologia, Universidade de Caxias do Sul, Caxias do Sul, Brazil

Objective: To evaluate the effect of cigarette smoking on antioxidant levels and the presence of leukocytospermia in infertile men.
Design: Prospective study.
Setting: Academic medical center.
Patient(s): Ten fertile donors and 112 infertile patients were included in the study.
Intervention(s): None.
Main Outcome Measure(s): Semen analysis was performed according to the World Health Organization guideline. The activity of the superoxide dismutase was based on the adrenochrome concentration, and the catalase activity was determined by the velocity of hydrogen peroxide consumption.
Result(s): Lower levels of superoxide dismutase and catalase were seen in infertile patients compared with fertile donors. Superoxide dismutase was significantly correlated with sperm concentration and negatively correlated with leukocytospermia. In addition, leukocytospermia was inversely correlated with sperm motility. Superoxide dismutase levels were negatively related to cigarette smoking.
Conclusion(s): Cigarette smoking may impair sperm motility and decrease the antioxidant activity (negative correlation with superoxide dismutase) in the seminal plasma. (Fertil Steril® 2008;90:278–83. ©2008 by American Society for Reproductive Medicine.)

Key Words: Cigarette, smoking, leukocytospermia, infertile, antioxidant, oxidative stress

Approximately 25% of infertility among couples can be attributed to diminished semen quality and other male factors (1). The etiology of diminished semen quality is generally poorly understood, although environmental, occupational, and lifestyle characteristics, such as age, alcohol, cigarette smoking, and diet, have been implicated (2–9).

Despite worldwide antismoking campaigns, cigarette smoking is very common. The highest prevalence of smoking is observed in young adult males during their reproductive period (46% smokers between 20 and 39 years) (10). In fact, approximately one third of the world's population age 15 years and older smokes cigarettes daily. According to the Brazilian National Institute of Cancer, between 31% and 42% of the Brazilian male population, varying from different regions of the country, are smokers (11). In addition, smoking among men is increasing in Central and Eastern Europe (12).

Numerous investigations have been conducted on the relationship between cigarette smoking and male infertility; however, the exact molecular mechanisms are not well understood in most cases (13). Cigarette smoking may be associated with subfertility in men and may result in decreased sperm concentration, lower sperm motility, and a reduced percentage of morphologically normal sperm (14). Smoking may damage the chromatin structure and produce endogenous DNA strand breaks in human sperm (15–17). In fact, levels of DNA damage tend to be higher in smokers (18). However, even though studies have shown that smoking has a detrimental effect on sperm quality, a handful of studies have found no association between smoking and sperm function or sperm nuclear DNA damage (19, 20). It has been described in rats that smoking leads to a secretory dysfunction of the Leydig cells and also a deficiency in sperm

Received September 23, 2007; revised January 22, 2008; accepted February 8, 2008.
F.F.P. has nothing to disclose. F.M.U. has nothing to disclose. M.S. has nothing to disclose. E.B. has nothing to disclose. B.P.S. has nothing to disclose. E.B.P. has nothing to disclose.
Supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) process no. 409651/2006-0 and Fundação de Amparo a Pesquisa no Rio Grande do Sul (Bolsa de iniciação cientifica).
Reprint requests: Fabio Firmbach Pasqualotto, M.D., Ph.D., Rua Pinheiro Machado, 2569, sl 23/24, Bairro São Pelegrino, Caxias do Sul, Rio Grande do Sul, Brazil 95020172 (FAX: 55-54-3215-1695; E-mail: fabio@conception-rs.com.br).

0015-0282/08/$34.00
doi:10.1016/j.fertnstert.2008.02.123

maturation and spermatogenesis (21). Cigarette smoke exposure results in diminished capacity of spermatozoa to penetrate oocytes. In addition, paternal cigarette smoke exposure affects the embryonic ability for implantation and has a detrimental effect on sperm fertilizing potentials in vivo and in vitro (21, 22). Furthermore, it appears that cotinine concentrations of 400 or 800 ng/mL exert a detrimental effect on sperm motility, membrane function, and the ability to undergo capacitation (23).

According to a meta-analysis including 27 studies on the association between cigarette smoking and semen quality, a mean reduction in sperm concentration of 13%, a mean reduction of sperm motility of 10%, and a mean reduction of morphologically normal sperm of 3% were reported in smokers (2). Most of the studies, however, that reported a significant difference in semen quality were performed in normal and healthy men not attending infertility clinics (10, 24, 25). Nevertheless, the majority of the studies included in that meta-analysis, which found detrimental effects of smoking on fertility, were performed in healthy volunteers, whereas only 25% of the studies were carried out in patients with suspected infertility. In fact, most studies had only a small number of samples (19). Some studies performed with a large number of samples could not find a relationship between cigarette smoking and seminal quality, although they were performed in patients with fertility problems (22, 23). However, it has been described that cigarette smoking is associated with reduced semen main parameters and sperm function tests that could worsen the male fertilizing potential, especially in borderline cases (26–28).

The mechanism behind the harmful effect of smoking on semen quality is not fully understood. Disturbance of the hypothalamo-pituitary-gonadal system (29) or mild hypoxia caused by the disruption of the testicular microcirculation (30) are possible explanations, but a direct toxic effect of the many chemical components in the cigarette smoke on the germinative epithelium is a more likely explanation (17). Oxidants in cigarette smoke are thought to damage sperm DNA, and smokers have more oxidative DNA damage in their sperm than do nonsmokers (16, 17, 31). An association between cigarette smoking and sperm aneuploidy has also been observed (32).

The greatest paradox of aerobic respiration is that oxygen, which is essential for energy production, may also be detrimental because it leads to the production of reactive oxygen species (ROS) (33). Controlled generation of ROS has a physiologic role in spermatozoal functions such as tyrosine kinase phosphorylation, hyperactivation, capacitation, and acrosome reaction (33). When levels of ROS overwhelm the body's antioxidant defense system, oxidative stress occurs. Sperm damage can be caused either by the invading pathogens or by the defense mechanisms that are employed against them (33). For example, when microorganisms invade the human body, it produces polymorphonuclear leukocytes and macrophages, which are the major sources of ROS production (34–39). Prostatitis and accessory gland infection increase oxidative stress, which severely damages spermatozoa (38, 39).

Antioxidants may play a critical role in protecting male germ cells against oxidative damage (40–44). Superoxide dismutase (SOD) and catalase are important antioxidant enzymes that can quench excess free radicals such as superoxide anion and hydrogen peroxide, respectively (43–45). The objective of our study was to evaluate the effect of cigarette smoking on antioxidant levels and the presence of leukocytospermia in infertile men.

MATERIALS AND METHODS

Institutional Review Board approval was obtained, and all patients gave informed consent by the time of the first appointment. A prospective analysis was performed in 10 fertile donors and 112 infertile patients. Male factor infertility was diagnosed when at least two successive semen analyses fulfilled the criteria of the World Health Organization (46). The evaluation of patients was performed by an urologist specialist in male factor infertility (F.F.P.). Patients with azoospermia or who were taking antioxidants were excluded from our study. Patients who requested a vasectomy for sterilization purposes were included in the study as a control group before the sterilization procedure. The laboratory evaluation for patients included at least two semen analyses obtained by masturbation after 2 to 5 days of abstinence with an interval of a month between them. Samples were collected into sterile containers, allowed to liquefy at 37°C for 30 minutes, and analyzed for sperm concentration, percent motility, and sperm morphology according to the World Health Organization (46).

Assays

Serum SOD activity was determined spectrophotometrically at 480 nm by measuring inhibition rate of autocatalytic adrenochrome formation. One SOD unit was defined as the amount of enzyme inhibiting the rate of adrenochrome formation by 50% per gram of protein (47). Catalase activity was assayed by the method of Maehly and Chance (48), with one unit of catalase decomposing 1 μmol of hydrogen peroxide per milligram of protein per minute at pH 7.4. Total protein was determined spectrophotometrically at 545 nm by the biuret method (Labtest Total Protein Kit; Labtest Diagnostica S.A., Lagoa Santa, Brazil).

White Blood Cells

The presence of granulocytes in semen specimens was assessed by a myeloperoxidase test (49). A 20-μL volume of liquefied specimen was placed in a Corning 2.0-mL cryogenic vial (Corning Costar Corp., Cambridge, MA); 20 μL of phosphate-buffered saline solution (pH 7.0) and 40 μL of benzidine solution were added. The mixture was vortexed and allowed to sit for 5 minutes. Five microliters then was placed on a Makler chamber (Sefi Medical, Haifa, Israel)

and examined for cells that had stained dark brown, indicating cells positive for peroxidase. Leukocytospermia was defined as least 1×10^6 white blood cells per milliliter.

Statistical Analysis

The variables were log transformed to normalize the data distribution. Comparisons between continuous variables were calculated with use of analysis of variance. Pearson's coefficient was used to evaluate correlation between SOD and catalase and other variables. A P value of $<.05$ was considered statistically significant. All statistical tests were performed with the Statistical Package for Social Sciences (version 10.0 for Windows; SPSS, Inc., Chicago, IL).

RESULTS

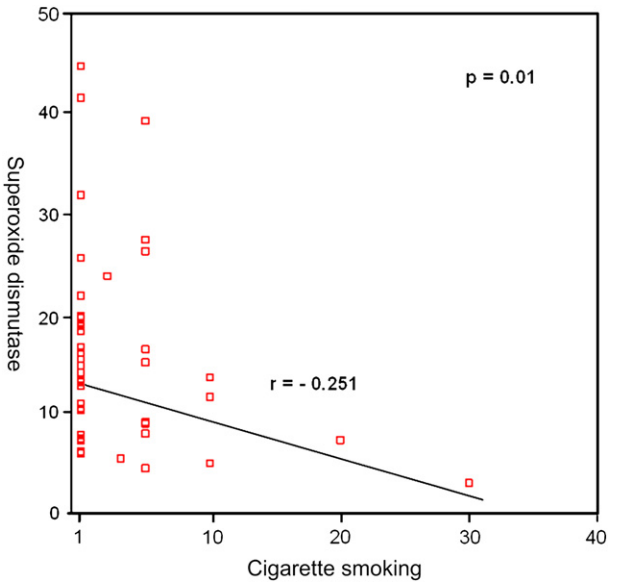
Sperm concentration ($\times 10^6$ sperm/mL) was higher in fertile donors (85.2 ± 41.2) than in infertile men (13.5 ± 6.9); P=.02. Sperm motility was higher in fertile donors (69.2% ± 23.6%) than in infertile men (41.1% ± 9.2%); P=.04. Sperm morphology was higher in fertile donors (31.2% ± 6.5%) than in infertile men (14.1% ± 9.1%); P=.04.

A statistically significant difference was noted in the number of cigarettes smoked per day between infertile (4.8 ± 0.9) and fertile men (1.5 ± 0.5; P=.03). Significantly lower levels of SOD (14.67 ± 12.27 USOD/G and 38.03 ± 21.65 USOD/G) and catalase (14.87 ± 16.95 UCAT/mg and 34.03 ± 20.65 UCAT/mg) activity were seen in infertile patients compared with fertile donors ($P<.0001$).

A significant correlation between catalase and SOD was observed ($r = 0.461$, P=.0001) (Table 1). Superoxide dismutase was significantly correlated with sperm concentration ($r = 0.204$, P=.034) and negatively correlated with leukocytospermia ($r = -0.228$, P=.021). In addition, leukocytospermia was inversely correlated with sperm motility ($r = -0.211$, P=.042). Cigarette smoking was inversely significantly correlated with SOD levels ($r = -0.251$, P=.01) (Fig. 1) but not with catalase levels ($r = -0.147$, P=.130) (Fig. 2). Leukocytospermia was related to cigarette smoking ($r = 0.311$, P=.009) (Fig. 3).

FIGURE 1

Correlation between SOD levels and cigarette consumption.



Pasqualotto. Antioxidants in semen of smokers. Fertil Steril 2008.

DISCUSSION

The negative impact of cigarette consumption occurs in spontaneous as well as in assisted reproduction (50, 51). For women, pregnancy rate is decreased, early spontaneous abortions are increased, and ovarian reserve is altered. For men, standard sperm parameters are modified, and spermatozoon nuclear quality is compromised. In fact, smokers' spermatozoa have a significantly higher DNA fragmentation than those of nonsmokers (51). In contrast, there is no statistically significant difference in conventional parameters between smokers and nonsmokers.

All living aerobic cells are normally exposed to some ROS, but if ROS levels rise oxidative stress occurs, which results in oxygen and oxygen-derived oxidants and in turn increases the

TABLE 1

Correlation between SOD and catalase with other variables.

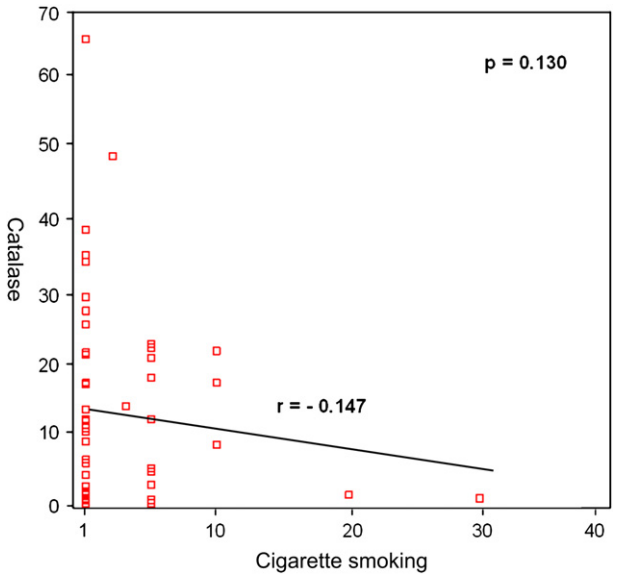
	SOD	Catalase	Cigarette smoking
Sperm concentration	$r = 0.204$, P=.03	$r = 0.144$, P=.165	$r = -0.187$, P=.09
Sperm motility	$r = 0.191$, P=.05	$r = 0.276$, P=.02	$r = -0.211$, P=.08
Sperm morphology	$r = 0.140$, P=.158	$r = 0.145$, P=.145	$r = -0.132$, P=.180
Leukocytospermia	$r = -0.228$, P=.021	$r = -0.125$, P=.35	$r = 0.311$, P=.009
SOD	—	$r = 0.461$, P=.0001	$r = -0.251$, P=.01
Catalase	$r = 0.461$, P=.0001	—	$r = -0.147$, P=.130

Note: $P<.05$ was considered significant.

Pasqualotto. Antioxidants in semen of smokers. Fertil Steril 2008.

FIGURE 2

Correlation between catalase levels and cigarette consumption.

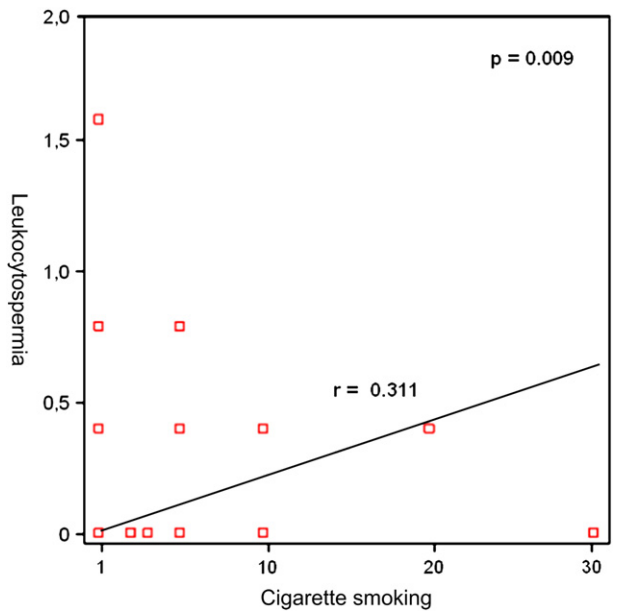


Pasqualotto. Antioxidants in semen of smokers. Fertil Steril 2008.

rates of cellular damage (40, 42). Oxidative stress has been shown to be a major cause of male infertility; a large proportion of infertile men have elevated levels of seminal ROS (52). Several forms of sperm DNA damage are caused by ROS, for example, chromatin cross-linking, chromosome deletion, DNA strand breaks, and base oxidation (53). Moreover, ROS are important in mediating apoptosis by inducing cytochrome c and caspases 9 and 3, which in turn result in a high frequency of single- and double-stranded DNA strand breaks (54). Therefore, in the context of male infertility, seminal oxidative stress, sperm DNA damage, and apoptosis are interlinked and constitute a unified pathogenic molecular mechanism (52, 53).

FIGURE 3

Correlation between leukocytospermia levels and cigarette consumption.



Pasqualotto. Antioxidants in semen of smokers. Fertil Steril 2008.

There are two main sources of ROS in semen: leukocytes and immature spermatozoa (55). Of these, leukocytes are considered to be the primary source (56). Leukocytes, particularly neutrophils and macrophages, have been associated with excessive ROS production that ultimately leads to sperm dysfunction (41, 42, 57, 58). Spermatozoa produce ROS mainly when a defect occurs during spermiogenesis that results in retention of cytoplasmic droplets (59). A strong positive correlation exists between immature spermatozoa and ROS production, which in turn negatively affects the sperm quality (44, 60). Environmental pollution and radiation can generate various ROS such as hydrogen peroxide (H_2O_2), superoxide anion (O_{2-}), and hydroxyl radical ($OH-$) (61). It has been described in tollgate workers that traffic pollution damages sperm and may reduce fertility in young and middle-aged men (62).

Smoking induces oxidative stress either by increasing levels of oxidants originating from the smoke or by decreasing levels of antioxidants in the seminal plasma (15, 18). Therefore, smokers may need to increase their intake of antioxidants to compensate for this deficiency (33). Other studies have reported that tobacco adversely affects sperm quality (i.e., concentration, motility, and morphology) (24). Smoking may also affect sperm DNA, as evidenced from the increased level of 8-deoxyguanosine, a marker for oxidative DNA damage (63). Therefore, smoking may not affect the fertilization rate per se, but it will increase the risk of heritable mutations (2, 64).

Several articles on the effects of cigarette consumption on human seminal quality have been published (13, 15, 17, 20, 24, 50, 51). Nevertheless, at present, there exists no consensus about the effects of these substances on male fertility (6, 9, 18, 41, 42, 65). The inconsistency in the literature seems to be due to the small sample size of most of the studies and differences in the population selected (healthy volunteers or patients with suspected infertility). At present, the effects of tobacco consumption on male reproductive function remain under discussion (9). Infertile men who smoke have higher levels of seminal oxidative stress than infertile nonsmokers (18). The link between cigarette smoking and high seminal ROS can be attributed in part to the associated increase in seminal leukocytes. Cigarette smoking seems to activate bone marrow, and it is speculated that blood leukocytes contribute to the chronic lung inflammation associated with cigarette smoking (66). The mechanism that activates leukocytes in the semen of smokers, however, is unclear.

Men with marginal semen quality who wish to have children might benefit from stopping smoking. Cigarette smoking is a cell mutagen and carcinogen and may adversely affect fertility. Every smoker should be encouraged to stop smoking, especially if a pregnancy is planned. Cigarette smoke contains a lot of known toxins, which may have detrimental effects on fertility in both sexes. Simply stopping smoking, however, could prevent the toxins contained in cigarette smoking.

The seminal plasma is endowed with an array of antioxidants that act as free radical scavengers to protect spermatozoa against oxidative stress (53). Seminal plasma contains a number of enzymatic antioxidants such as SOD, catalase, and glutathione peroxidase. In addition, it contains a variety of nonenzymatic antioxidants such as vitamin C (ascorbic acid), vitamin E (α-tocopherol), pyruvate, glutathione, and carnitine (67). Rational strategies of reducing the seminal oxidative stress may be effective for the treatment of male infertility when the free radicals are produced in excess, for instance, in reproductive tract infections. Because our study showed a negative correlation between SOD and cigarette smoking, augmentation of the scavenging capacity of the seminal plasma by supplementation with antioxidants may be useful.

Although several clinical intervention trials of antioxidants have found improvement in semen characteristics, the health and habits of the men in our study may also contribute to differences in findings from those of some of the clinical trials. Rolf et al. hypothesized that the length of vitamin C and E administration in their study (i.e., 8 weeks) may have been too short to improve semen quality in infertile men if the effect is on the testis (68).

Ascorbic acid concentration in the seminal plasma has been found to be negatively associated with ROS activity in sperm of infertile men, and the depletion of ascorbic acid intake has been associated with an increase in oxidative damage in the sperm of healthy men (15). The beneficial effect of vitamin C oral administration on sperm quality has been documented in smokers. A significant positive correlation has been observed between serum, seminal plasma vitamin C concentrations, and sperm quality (69). Smokers normally need two to three times the intake of vitamin C to maintain blood plasma levels comparable with those of nonsmokers, and some studies include both smokers and nonsmokers (15, 70).

Therefore, in our study, we can conclude that decreased antioxidant enzyme levels are associated with male infertility, and the detrimental effects of cigarette smoking on sperm motility and antioxidant levels (negative correlation with SOD) may be a possible reason for infertility in men who smoke cigarettes. Given the potential adverse effects of low antioxidant levels on fertility, physicians should advise infertile patients who smoke cigarettes to quit smoking.

REFERENCES

1. Templeton A. Infertility—epidemiology, aetiology and effective management. Health Bull 1995;53:294–8.
2. Vine MF. Smoking and male reproduction: a review. Int J Androl 1996;19:323–37.
3. Weber RFA, Pierik FH, Dohle GR, Burdorf A. Environmental influences on male reproduction. BJU Int 2002;89:143–8.
4. Auger J, Eustache F, Andersen AG, Irvine DS, Jorgensen N, Skakkebaek NE, et al. Sperm morphological defects related to environment, lifestyle and medical history of 1001 male partners of pregnant women from four European cities. Hum Reprod 2001;16:2710–7.
5. Eskenazi B, Wyrobek AJ, Sloter E, Kidd SA, Moore L, Young S, et al. The association of age and semen quality in healthy men. Hum Reprod 2003;18:447–54.
6. DeMarini DM. Genotoxicity of tobacco smoke and tobacco smoke condensate: a review. Mutation Research 2004;567:447–74.
7. Pasqualotto FF, Lucon AM, Sobreiro BP, Pasqualotto EB, Arap S. Effects of medical therapy, alcohol, smoking and endocrine disruptors on male infertility. Rev Hosp Clin Fac Med S Paulo 2004;59:375–82.
8. Pasqualotto FF, Sobreiro BP, Hallak J, Pasqualotto EB, Lucon AM. Cigarette smoking is related to a decrease in semen volume in a population of fertile men. BJU Int 2006;97:324–6.
9. Ramlau-Hansen CH, Thulstrup AM, Aggerholm ASA, Jensen MS, Toft G, Bonde JP. Is smoking a risk factor for decreased semen quality? A cross-sectional analysis. Hum Reprod 2007;22:188–96.
10. Trummer H, Habermann H, Haas J, Pummer K. The impact of cigarette smoking on human semen parameters and hormones. Hum Reprod 2002;17:1554–9.
11. National Institute of Cancer, Brazil Ministry of Health. Tobacco control. Available from: URL: http://www.inca.gov.br/tabagismo/.
12. Centers for Disease Control and Prevention. Behavioral risk factor surveillance system survey data. Atlanta (GA): US Department of Health and Human Services, Centers for Disease Control and Prevention, 2002.
13. Arabi M, Mosthaghi H. Influence of cigarette smoking on spermatozoa via seminal plasma. Andrologia 2005;37:119–24.
14. Sofikitis N, Miyagawa I, Dimitriadis D, Zavos P, Sikka S, Hellstrom W. Effects of smoking on testicular function: semen quality and sperm fertilizing capacity. J Urol 1995;154:1030–4.
15. Fraga CG, Motchnik PA, Wyrobek AJ, Rempel DM, Ames BN. Smoking and low antioxidant levels increase oxidative damage to sperm DNA. Mutat Res 1996;351:199–203.
16. Shen HM, Chia SE, Ni ZY, New AL, Lee BL, Ong CN. Detection of oxidative DNA damage in human sperm and the association with cigarette smoking. Reprod Toxicol 1997;11:675–80.
17. Zenzes MT. Smoking and reproduction: gene damage to human gametes and embryos. Hum Reprod Update 2000;6:122–31.
18. Saleh RA, Agarwal A, Sharma RK, Nelson DR, Thomas AJ Jr. Effect of cigarette smoking on levels of seminal oxidative stress in infertile men: a prospective study. Fertil Steril 2002;78:491–9.
19. Sergerie M, Ouhilal S, Bissonnette F, Brodeurand J, Bleau G. Lack of association between smoking and DNA fragmentation in spermatozoa of normal men. Hum Reprod 2000;15:1314–21.
20. Vogt HJ, Heller WD, Borelli S. Sperm quality of health smokers, ex-smokers, and never smokers. Fertil Steril 1986;45:106–10.
21. Yamamoto Y, Isoyama E, Sofikitis N, Miyagawa I. Effects of smoking on testicular function and fertilizing potential in rats. Urol Res 1998;26: 45–8.
22. Kapawa A, Giannakis D, Tsoukanelis K, Kanakas N, Baltogiannis D, Agapitos E, et al. Effect of paternal cigarette smoking on testicular function, sperm fertilizing capacity, embryonic development, and blastocyst capacity form implantation in rats. Andrologia 2004;36:57–68.
23. 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;28:370–5.
24. 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;19:287–91.
25. Sobreiro BP, Lucon AM, Pasqualotto FF, Hallak H, Athayde KS, Arap S. Semen analysis in fertile patients undergoing vasectomy in the Hospital das Clinicas of the São Paulo State University: values of reference and variations according to age, period of sexual abstinence, period of the

year, smoking habits and consumption of caffeine. São Paulo Med J 2005;123:161–6.
26. Kiziler AR, Aydemir B, Onaran I, Alici B, Ozkara H, Gulyasar T, et al. High levels of cadmium and lead in seminal fluid and blood of smoking men are associated with high oxidative stress and damage in infertile subjects. Biol Trace Elem Res 2007;120:82–91.
27. Mostafa T, Tawadrous G, Roaia MM, Amer MK, Kader RA, Aziz A. Effect of smoking on seminal plasma ascorbic acid in infertile and fertile males. Andrologia 2006;38:221–4.
28. Dikshit RK, Buch JG, Mansuri SM. Effect of tobacco consumption on semen quality of a population of hypofertile males. Fertil Steril 1987;48:334–6.
29. Vermeulen A. Environment, human reproduction, menopause, and andropause. Environ Health Perspect 1993;101:91–100.
30. Collin O, Kilter S, Bergh A. Tobacco smoke disrupts testicular microcirculation in the rat. Int J Androl 1995;18:141–5.
31. Horak S, Polanska J, Widlak P. Bulky DNA adducts in human sperm: relationship with fertility, semen quality, smoking, and environmental factors. Mutat Res 2003;537:53–65.
32. Shi Q, Ko E, Barclay L, Hoang T, Rademaker A, Martin R. Cigarette smoking and aneuploidy in human sperm. Mol Reprod Dev 2001;59: 417–21.
33. Saleh RA, Agarwal A. Oxidative stress and male infertility: from research bench to clinical practice. J Androl 2002;23:737–52.
34. Ochsendorf FR. Infections in the male genital tract and reactive oxygen species. Hum Reprod Update 1999;5:399–420.
35. Saran M, Beck-Speier I, Fellerhoff B, Bauer G. Phagocytic killing of microorganisms by radical processes: consequences of the reaction of hydroxyl radicals with chloride yielding chlorine atoms. Free Radic Biol Med 1999;26:482–90.
36. Zalata AA, Ahmed AH, Allamaneni SS, Comhaire FH, Agarwal A. Relationship between acrosin activity of human spermatozoa and oxidative stress. Asian J Androl 2004;6:313–8.
37. Shefi S, Turek PJ. Definition and current evaluation of subfertile men. Int Braz J Urol 2006;32:385–97.
38. Pasqualotto FF, Sharma RK, Potts JM, Nelson DR, Thomas AJ, Agarwal A. Seminal oxidative stress in patients with chronic prostatitis. Urology 2000;55:881–5.
39. Potts JM, Pasqualotto FF. Seminal oxidative stress in patients with chronic prostatitis. Andrologia 2003;35:304–8.
40. Sharma RK, Pasqualotto FF, Nelson DR, Thomas AJ Jr, Agarwal A. ROS-TAC is a novel marker of oxidative stress. Hum Reprod 1999;14: 2801–7.
41. Sharma RK, Pasqualotto FF, Nelson DR, Thomas AJ, Agarwal A. Role of leukocytospermia in oxidative stress. J Androl 2001;22:575–83.
42. Pasqualotto FF, Sharma RK, Nelson DR, Thomas AJ Jr, Agarwal A. Relationship between oxidative stress, semen characteristics and clinical diagnosis in men undergoing infertility investigation. Fertil Steril 2000;73:459–64.
43. Zini A, de Lamirande E, Gagnon C. Reactive oxygen species in semen of infertile patients: levels of superoxide dismutase- and catalase-like activities in seminal plasma and spermatozoa. Int J Androl 1993;16: 183–8.
44. Said RM, Agarwal A, Sharma RK, Mascha E, Sikka S, Thomas AJ Jr. Human sperm superoxide anion generation and correlation with semen quality in patients with male infertility. Fertil Steril 2004;82:871–4.
45. Agarwal A, Gupta S, Sikka S. The role of free radicals and antioxidants in reproduction. Curr Opin Obstet Gynecol 2006;18:325–32.
46. World Health Organization. Laboratory manual for the examination of human semen and semen-cervical mucus interaction. 4th ed. New York: Cambridge University Press, 1999.
47. Misra HP, Fridovich I. The generation of superoxide radical during autoxidation of hemoglobin. J Biol Chem 1972;247:6960–2.
48. Maehly AC, Chance B. The assay of catalases and peroxidases. Methods Biochem Anal 1954;1:357–424.
49. Shekarriz M, Sharma RK, Thomas AJ, Agarwal A. Positive myeloperoxidase staining (Endtz test) as an indicator of excessive reactive oxygen species formation in semen. J Assist Reprod Genet 1995;12:70–4.
50. Sepaniak S, Forges T, Monnier-Barbarino P. Cigarette smoking and fertility in women and men. Gynecol Obstet Fertil 2006;34:945–9.
51. Sepaniak S, Forges T, Gerard H, Folliguet B, Bene MC, Monnier-Barbarino P. The influence of cigarette smoking on human sperm quality and DNA. Toxicology 2006;223:54–60.
52. Agarwal A, Said TM. Oxidative stress, DNA damage and apoptosis in male infertility: a clinical approach. BJU Int 2005;95:503–7.
53. Agarwal A, Saleh RA, Bedaiwy MA. Role of reactive oxygen species in the pathophysiology of human reproduction. Fertil Steril 2003;79: 829–43.
54. Said T, Paasch U, Glander H, Agarwal A. Role of caspases in male infertility. Hum Reprod Update 2004;10:39–51.
55. 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;6:59–65.
56. Aitken RJ, Buckingham D, West K, Wu FC, Zikopoulos K, Richardson DW. Differential contribution of leucocytes and spermatozoa to the generation of reactive oxygen species in the ejaculates of oligozoospermic patients and fertile donors. J Reprod Fertil 1992;94:451–62.
57. Aitken RJ, Baker HW. Seminal leukocytes: passengers, terrorists or good samaritans? Hum Reprod 1995;10:1736–9.
58. Aitken RJ, Fisher HM, Fulton N, Gomez E, Knox W, Lewis B, et al. Reactive oxygen species generation by human spermatozoa is induced by exogenous NADPH and inhibited by the flavoprotein inhibitors diphenylene iodonium and quinacrine. Mol Reprod Dev 1997;47:468–82.
59. Gomez E, Buckingham DW, Brindle J, Lanzafame F, Irvine DS, Aitken RJ. Development of an image analysis system to monitor the retention of residual cytoplasm by human spermatozoa: correlation with biochemical markers of the cytoplasmic space, oxidative stress, and sperm function. J Androl 1996;17:276–87.
60. Gil-Guzman E, Ollero M, Lopez MC, Sharma RK, Alvarez JG, Thomas AJ Jr, et al. Differential production of reactive oxygen species by subsets of human spermatozoa at different stages of maturation. Hum Reprod 2001;16:1922–30.
61. Gate L, Paul J, Ba GN, Tew KD, Tapiero H. Oxidative stress induced in pathologies: the role of antioxidants. Biomed Pharmacother 1999;53: 169–80.
62. De Rosa M, Zarrilli S, Paesano L, Carbone U, Boggia B, Petretta M, et al. Traffic pollutants affect fertility in men. Hum Reprod 2003;18:1055–61.
63. Potts RJ, Newbury CJ, Smith G, Notarianni LJ, Jefferies TM. Sperm chromatin damage associated with male smoking. Mutat Res 1999;423:103–11.
64. Rubes J, Lowe X, Moore D, Perreault S, Slott V, Evenson D, et al. Smoking cigarettes is associated with increased sperm disomy in teenage men. Fertil Steril 1998;70:715–23.
65. Navas-Acien A, Peruga A, Breysse P, Zavaleta A, Blanco-Marquizo A, Pitarque R, et al. Secondhand tobacco smoke in public places in Latin America, 2002–2003. JAMA 2004;291:2741–5.
66. van Eeden SF, Hogg JC. The response of human bone marrow to chronic cigarette smoking. Eur Respir J 2000;15:915–21.
67. Agarwal A, Nallella KP, Allamaneni SSR, Said TM. Role of antioxidants in treatment of male infertility: an overview of the literature. Reprod Biomed Online 2004;8:616–27.
68. Rolf C, Cooper TG, Yeung CH, Nieschlag E. Antioxidant treatment of patients with asthenozoospermia or moderate oligoasthenozoospermia with high-dose vitamin C and vitamin E: a randomized, placebo-controlled, double-blind study. Hum Reprod 1999;14:1028–33.
69. Dawson EB, Harris WA, Teter MC, Powell LC. Effect of ascorbic acid supplementation on the sperm quality of smokers. Fertil Steril 1992;58:1034–8.
70. Comhaire FH, Mahmoud A. The role of food supplementation in the treatment of the infertile man. Reprod Biomed Online 2003;7:385–91.