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Reduction of the Incidence of Sperm DNA Fragmentation by Oral Antioxidant Treatment

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ABSTRACT: Sperm DNA fragmentation is known to compromise male fertility. Previous findings have suggested the implication of oxidative stress in the etiology of this pathological condition. The present study was conducted to find out if the pathologically increased incidence of DNA fragmentation in ejaculated spermatozoa can be reduced by oral treatment with two antioxidants, vitamins C and E. Sixty-four men with unexplained infertility and an elevated (≥15%) percentage of DNA-fragmented spermatozoa in the ejaculate were randomized between an antioxidant treatment (1 g vitamin C and 1 g vitamin E daily for 2 months) group and a placebo group. Sperm DNA fragmentation was evaluated by terminal deoxyribonucleotidyl transferase–mediated dUTP nick-end labeling assay before and after treatment. No differences in basic sperm parameters were found between the antioxidant treatment and the placebo group before or after treatment. However, the percentage of DNA-fragmented spermatozoa was markedly reduced ($P < .001$) in the antioxidant treatment group after the treatment (9.1 ± 7.2) as compared with the pretreatment values (22.1 ± 7.7). No difference in the pretreatment and posttreatment incidence of sperm DNA fragmentation was observed in the placebo group. These data show that sperm DNA damage can be efficiently treated with oral antioxidants administered during a relatively short time period.

Key words: Sperm DNA damage, ejaculated spermatozoa, TUNEL assay, in vivo treatment, vitamin C, vitamin E.

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Excessive sperm DNA damage has been shown to compromise male fertility (Evenson et al, 1980, 1999; Aitken, 1999; Carrel et al, 2003; Henkel et al, 2004; Tesarik et al, 2004a) and may predispose to genetic diseases, birth defects, and childhood cancer in the offspring (Fraga et al, 1996; Ji et al, 1997; Aitken et al, 2003). Several studies have pointed out a central role of oxidative stress, leading to the formation of reactive oxygen species, in the etiology of sperm DNA damage (Sikka et al, 1995; Twigg et al, 1998; Aitken and Krausz, 2001; Saleh and Agarwal, 2002; Aitken et al, 2003; Moustafa et al, 2004). These findings extended earlier original observations on the role of oxidative stress in the etiology of male infertility (Jones et al, 1979; Aitken and Clarkson, 1987). In vitro studies have demonstrated a protective effect of several antioxidants on sperm DNA integrity (Griveau and Le Lannou, 1994; Hughes et al, 1998; Lopes et al, 1998; Twigg et al, 1998; Donnelly et al, 1999; Dobrzynska et al, 2004).

An improvement of basic sperm parameters by oral treatment with antioxidants has been reported in a number of studies (reviewed in Agarwal, 2004), but DNA damage has been addressed in only a few of them (Geva et al, 1996; Suleiman et al, 1996; Kodama et al, 1997; Comhaire et al, 2000; Keskes-Ammar et al, 2003). Only two of these studies were controlled and randomized (Suleiman et al, 1996; Keskes-Ammar et al, 2003), and none of them used a direct assay for the detection of DNA strand breaks.

This study was designed as a prospective, placebo-controlled and double-blinded analysis of the in vivo effects of antioxidants, administered orally for 2 months in men with previously diagnosed high incidence of sperm DNA fragmentation, on the percentage of spermatozoa carrying fragmented DNA. DNA fragmentation was assessed by terminal deoxyribonucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) assay. In parallel, the effects of the treatment on basic sperm parameters in this group of patients were also evaluated.

Materials and Methods

Study Design and Participants

This study involved 64 men consulting for infertility in whom previously performed TUNEL assay showed the presence of fragmented DNA in more than or equal to 15% of ejaculated spermatozoa. Patients with varicocele, genitourinary inflammation, or infection, and smokers were excluded. The study participants were prospectively randomized into 2 groups. Members

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Table 1. *Basic sperm parameters and the incidence of TUNEL-positive spermatozoa in the patients randomized between the antioxidant-treatment and the placebo group**

Group	Sperm Concentration	Percent: Sperm Motility	Percent: Normal Sperm Forms	Percent: TUNEL-Positive Spermatozoa
Antioxidant	18.8 ± 17.2	40.2 ± 25.1	10.5 ± 8.1	22.1 ± 7.7
Placebo	19.1 ± 17.4	39.9 ± 24.8	11.0 ± 8.2	22.4 ± 7.8

* Data are mean ± SD. TUNEL indicates terminal deoxyribonucleotidyl transferase–mediated dUTP nick-end labeling.

of one group were given a 2-month oral treatment with 2 antioxidants, vitamin C and vitamin E, both at a daily dose of 1 g (500 mg twice a day). Members of the other group received a placebo during the same period. Basic sperm parameters and the incidence of sperm DNA fragmentation were evaluated in both groups before and after the treatment period. The study was double-blinded, with both the authors and the patients unaware of which of the patients was in the treatment or control arm of the study.

Evaluation of Basic Sperm Parameters

Basic sperm parameters, including sperm count, concentration, motility, and morphology, were evaluated according to World Health Organization recommendations (World Health Organization, 1999).

Evaluation of Sperm DNA Fragmentation

Fragmented DNA in spermatozoa was visualized by TUNEL using the Cell Death Detection Kit with tetramethylrhodamine-labeled dUTP (Roche, Monza, Italy) and according to the manufacturer's instructions. Ejaculated sperm samples were washed from seminal plasma by low-speed centrifugation (200×; 10 minutes), smeared on microscope slides, air-dried, fixed with 4% paraformaldehyde in phosphate-buffered saline at 4°C for 25 minutes, and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. Fixed sperm smears were further processed for TUNEL as described (Tesarik et al, 2004a). Spermatozoa with fragmented DNA were detected in an epifluorescence microscope with a ×100 oil immersion objective. For quantitative evaluation, 500 spermatozoa in 50 randomly selected areas on 3 different microscope slides were evaluated for each sample, and the percentage of TUNEL-positive spermatozoa was determined.

Statistical Analysis

Differences between groups were assessed by 2-tailed *2* test with Yates correction and Fisher exact test. All analyses were performed using the Statistica 5.0 package (Statsoft Version 5.1, Hamburg, Germany).

Results

Basic sperm parameters, including sperm count (not shown), sperm concentration, motility, and the percentage of normal sperm forms showed a high interindividual variability in both the antioxidant treatment and the placebo group, but no significant differences in any of these parameters were found between the two groups (Table 1). The presence of fragmented DNA was detected by TUNEL in more than or equal to 15% of spermatozoa in all participants of this study before the beginning of treatment, and this percentage was not significantly different between the antioxidant treatment and the placebo group (Table 1).

After 2 months of antioxidant treatment, no significant change in sperm count (not shown), concentration, motility, and morphology was found in either the antioxidant treatment or the placebo group (Table 2). However, there was a slight trend toward a higher sperm concentration ($P = .12$) and a lower percentage of normal sperm forms ($P = .19$) after the 2-month treatment period, compared with the pretreatment situation. In fact, 20 out of the 32 patients in the antioxidant treatment group showed an increase in sperm concentration, and 19 of these patients showed a decrease in the percentage of normal sperm forms after 2 months of treatment.

In contrast to basic sperm parameters, the percentage of spermatozoa with fragmented DNA was significantly reduced in patients receiving antioxidant treatment for 2

Table 2. *Comparison of basic sperm parameters and the incidence of DNA fragmentation in the antioxidant treatment group before and after the treatment period**

Time of Analysis	Sperm Concentration (×10⁶/mL)	Percent: Sperm Motility	Percent: Normal Sperm Forms	Percent: TUNEL-Positive Spermatozoa
Before treatment	18.8 ± 17.2†	40.2 ± 25.1†	10.5 ± 8.1†	22.1 ± 7.7‡
After treatment	27.5 ± 24.6†	41.6 ± 22.0†	8.0 ± 7.1†	9.1 ± 7.2‡

* Data are mean ± SD. TUNEL indicates terminal deoxyribonucleotidyl transferase–mediated dUTP nick-end labeling.
† $P > .05$.
‡ $P < .001$.

Table 3. *Comparison of basic sperm parameters and the incidence of DNA fragmentation in the placebo group before and after the treatment period**

Time of Analysis	Sperm Concentration ($\times 10^6$/mL)	Percent		
		Sperm Motility	Normal Sperm Forms	TUNEL-Positive Spermatozoa
Before treatment	19.1 ± 17.4†	39.9 ± 24.8†	11.0 ± 8.2†	22.4 ± 7.8†
After treatment	20.3 ± 21.2†	38.7 ± 21.5†	11.6 ± 7.8†	22.9 ± 7.9†

* Data are mean ± SD. TUNEL indicates terminal deoxyribonucleotidyl transferase–mediated dUTP nick-end labeling.
† $P > .05$.

months as compared with the placebo group (Table 2). In fact, the incidence of DNA fragmentation was lower in all of the 32 men involved in this group after the antioxidant treatment, compared with the pretreatment values.

Patients involved in the placebo group did not show any significant changes in any of the sperm parameters tested, including sperm count, concentration, motility, morphology, and the incidence of DNA fragmentation after 2 months of treatment as compared with the pretreatment figures (Table 3). When data are represented as differences between the values before and after the 2-month treatment period, a significant difference between the antioxidant treatment and the placebo group was also found in the frequency of DNA fragmentation only, although there was a trend toward a higher sperm concentration and a lower percentage of normal sperm forms in the treatment group as compared with the placebo group (Table 4).

Discussion

The present data show a significant reduction in the incidence of DNA fragmentation in ejaculated spermatozoa after 2 months of oral antioxidant treatment. This is the first direct demonstration of an in vivo protective effect of antioxidants upon sperm DNA integrity, although such an effect has previously been suggested with the use of indirect assays (Geva et al, 1996; Suleiman et al, 1996; Kodama et al, 1997; Comhaire et al, 2000; Keskes-Ammar et al, 2003). We have shown previously that germ cells from men with different testiculopathies suffer DNA damage mainly after release from Sertoli cells, which normally occurs at a late elongated spermatid stage but may be shifted toward earlier stages of spermatogenesis under pathological conditions (Tesarik et al, 2004b). However, prematurely released immature germ cells are unlikely to develop into spermatozoa (Tesarik et al, 2004b). Because the duration of treatment in our study covered most of the spermatogenic cycle, no new conclusion about when the oxidative damage occurred in these patients can be drawn.

The present data are compatible with the hypothesis of posttesticular mechanism of sperm DNA damage (Tesarik et al, 2004b). However, they do not exclude the possibility that in vivo antioxidant treatment may act during the testicular period of germ cell development by exerting a beneficial effect on germ cells, Sertoli cells, or both, leading to a better function of defense mechanisms that will protect germ cells and spermatozoa against DNA damage.

In contrast to the marked effects on sperm DNA integrity, our data do not show any significant improvement of sperm concentration, motility, and morphology after in vivo antioxidant treatment. Previously published data on the effects of antioxidants on sperm concentration, motility, and morphology are contradictory (reviewed in Agarwal, 2004). The differences between individual studies are likely to be related to the type and dose of the antioxidant used, the characteristics of the patient group under treatment, and the duration of the treatment. As to the combination of vitamins C and E, our observations agree with those of Kodama et al (1997) and Rolf et al (1999), who also failed to observe an improvement in basic sperm parameters. In contrast, an improvement of

Table 4. *Comparison of differences in basic sperm parameters and the incidence of DNA fragmentation in the antioxidant treatment and the placebo groups after the treatment period**

Group	Percent			
	Sperm Concentration	Sperm Motility	Normal Sperm Forms	TUNEL-Positive Spermatozoa
Antioxidant	53.4 ± 95.5†	2.9 ± 10.8†	−21.1 ± 133.9†	−58.4 ± 27.8‡
Placebo	12.2 ± 74.3†	6.0 ± 13.3†	−4.4 ± 24.9†	6.6 ± 12.1‡

* Data are mean ± SD. TUNEL indicates terminal deoxyribonucleotidyl transferase–mediated dUTP nick-end labeling.
† $P > .05$.
‡ $P < .001$.

sperm concentration and motility has been described after 6-month treatment with vitamin E alone (Suleiman et al, 1996) and after 3-month treatment with vitamin E and selenium (Keskes-Ammar et al, 2003), and another group (Scott et al, 1998) reported an improvement of sperm motility after 3-month treatment with a combination of selenium with vitamins A, C, and E. The discrepancies between these observations may also be due to different pathophysiological backgrounds predominating in each of these studies. It is possible that oxidative damage may be involved in the etiology of defective spermatogenesis in some patients and not in others.

From the clinical point of view, the present findings open the question of whether male infertility associated with sperm DNA damage can be alleviated by antioxidant treatment. High levels of sperm DNA damage have been reported to decrease male fertility (Evenson et al, 1980, 1999; Aitken, 1999; Carrel et al, 2003; Henkel et al, 2004; Tesarik et al, 2004a), and it is thus reasonable to prescribe antioxidant treatment in such patients, provided that their basic sperm parameters are compatible with in vivo conception. In fact, some studies have reported an improvement of pregnancy rates in asthenozoospermic patients after combined oral antioxidant treatments (Suleiman et al, 1996; Scott et al, 1998; Comhaire et al, 2000; Vicari and Calogero, 2000; Vicari et al, 2002; Lenzi et al, 2003).

If basic sperm parameters are hardly compatible with in vivo conception, antioxidant treatment may be considered to increase the chance of an assisted reproduction attempt. As in natural conception, assisted reproduction outcomes have been shown to be negatively influenced by sperm DNA fragmentation (Host et al, 2000; Larson et al, 2000; Morris et al, 2002; Tomsu et al, 2002; Benchaib et al, 2003; Larson-Cook et al, 2003). According to one study, this effect is enhanced when intracytoplasmic sperm injection (ICSI) is used to assist in vitro fertilization (IVF), compared to conventional IVF (Benchaib et al, 2003). Study is under way to evaluate the effect of antioxidant treatment on ICSI outcomes in patients with high levels of sperm DNA fragmentation combined with severe oligoasthenoteratozoospermia.

In conclusion, this study shows that in infertile nonsmoking men with unexplained high levels of DNA fragmentation, the percentage of DNA-fragmented spermatozoa in the ejaculate can be very efficiently reduced by a relatively short oral treatment with a combination of vitamins C and E. The possible impact of this treatment on the patient's fertility status and on the fertilizing ability of spermatozoa in assisted reproductive technologies remain to be evaluated.

References

Aitken RJ. The Amoroso Lecture. The human spermatozoon—a cell in crisis? *J Reprod Fertil.* 1999;115:1–7.

Aitken RJ, Baker MA, Sawyer D. Oxidative stress in the male germ line and its role in the aetiology of male infertility and genetic disease. *Reprod Biomed Online.* 2003;7:65–70.

Aitken RJ, Clarkson JS. Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. *J Reprod Fertil.* 1987;83:459–469.

Aitken RJ, Krausz C. Oxidative stress, DNA damage and the Y chromosome. *Reproduction.* 2001;122:497–506.

Benchaib M, Braun V, Lornage J, Hadj S, Salle B, Lejeune H, Guérin JF. Sperm DNA fragmentation decreases the pregnancy rate in an assisted reproductive technique. *Hum Reprod.* 2003;18:1023–1028.

Carrell DT, Liu L, Peterson CM, Jones KP, Hatasaka HH, Erickson L, Campbell B. Sperm DNA fragmentation is increased in couples with unexplained recurrent pregnancy loss. *Arch Androl.* 2003;49:49–55.

Comhaire FH, Christophe AB, Zalata AA, Dhooge WS, Mahmoud AM, Depuydt CE. The effects of combined conventional treatment, oral antioxidants and essential fatty acids on sperm biology in subfertile men. *Prostaglandins Leukot Essent Fatty Acids.* 2000;63:159–165.

Dobrzynska MM, Baumgartner A, Anderson D. Antioxidants modulate thyroid hormone- and noradrenalin-induced DNA damage in human sperm. *Mutagenesis.* 2004;19:325–330.

Donnelly ET, McClure N, Lewis SE. The effect of ascorbate and alpha-tocopherol supplementation in vitro on DNA integrity and hydrogen peroxide–induced DNA damage in human spermatozoa. *Mutagenesis.* 1999;14:505–512.

Evenson DP, Darzynkiewicz Z, Melamed MR. Relation of mammalian sperm chromatin heterogeneity to fertility. *Science.* 1980;240:1131–1133.

Evenson DP, Jost LK, Marshall D, Zinaman MJ, Clegg E, Purvis K, De Angelis P, Clausen OP. Utility of the sperm chromatin structure assay (SCSA) as a diagnostic and prognostic tool in the human fertility clinic. *Hum Reprod.* 1999;14:1039–1049.

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.

Geva E, Lessing JB, Lerner-Geva L, Amit A. Free radicals, antioxidants and human spermatozoa: clinical implications. *Hum Reprod.* 1998;13:1422–1424.

Griveau JF, Le Lannou D. Effects of antioxidants on human sperm preparation techniques. *Int J Androl.* 1994;17:225–231.

Henkel R, Hajimohammad M, Stalf T, et al. Influence of deoxyribonucleic acid damage on fertilization and pregnancy. *Fertil Steril.* 2004;81:965–972.

Host E, Lindenberg S, Smidt-Jensen S. The role of DNA strand breaks in human spermatozoa used for IVF and ICSI. *Acta Obstet Gynecol Scand.* 2000;79:559–563.

Hughes CM, Lewis SE, McKelvey-Martin VJ, Thompson W. The effects of antioxidant supplementation during Percoll preparation on human sperm DNA integrity. *Hum Reprod.* 1998;13:1240–1247.

Ji BT, Shu XO, Linet MS, Zheng W, Wacholder S, Gao YT, Ying DM, Jin F. Paternal cigarette smoking and the risk of childhood cancer among offspring of nonsmoking mothers. *J Natl Cancer Inst.* 1997;89:238–244.

Jones R, Mann T, Sherins RJ. Peroxidative breakdown of phospholipids in human spermatozoa: spermicidal effects of fatty acid peroxides, and protective action of seminal plasma. *Fertil Steril.* 1979;31:531–537.

Keskes-Ammar L, Feki-Chakroun N, Rebai T, 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;49:83–94.

Kodama H, Yamaguchi R, Fukuda J, Kasai H, Tanaka T. Increased oxidative deoxyribonucleic acid damage in the spermatozoa of infertile male patients. *Fertil Steril.* 1997;68:519–524.

Larson KL, DeJonge CJ, Barnes AM, Jost LK, Evenson DP. Sperm chro-

matin structure assay parameters as predictors of failed pregnancy following assisted reproductive techniques. *Hum Reprod.* 2000;15: 1717–1722.

Larson-Cook KL, Brannian JD, Hansen KA, Kasperson KM, Aamold ET, Evenson DP. Relationship between the outcomes of assisted reproductive techniques and sperm DNA fragmentation as measured by the sperm chromatin structure assay. *Fertil Steril.* 2003;80:895–902.

Lenzi A, Lombardo F, Sgro P, Salacone P, Caponecchia L, Dondero F, Gandini L. Use of carnitine therapy in selected cases of male factor infertility: a double-blind crossover trial. *Fertil Steril.* 2003;79:292–300.

Lopes S, Sun JG, Jurisicova A, Meriano J, Casper RF. Sperm deoxyribonucleic acid fragmentation is increased in poor-quality semen samples and correlates with failed fertilization in intracytoplasmic sperm injection. *Fertil Steril.* 1998;69:528–532.

Morris ID, Ilott S, Dixon L, Brison DR. The spectrum of DNA damage in human sperm assessed by single cell gel electrophoresis (Comet assay) and its relationship to fertilization and embryo development. *Hum Reprod.* 2002;17:990–998.

Moustafa MH, Sharma RK, Thornton J, Mascha E, Abdel-Hafez MA, Thomas AJ Jr, Agarwal A. Relationship between ROS production, apoptosis and DNA denaturation in spermatozoa from patients examined for infertility. *Hum Reprod.* 2004;19:129–138.

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 study. *Hum Reprod.* 1999;14:1028–1033.

Saleh RA, Agarwal A. Oxidative stress and male infertility: from research bench to clinical practice. *J Androl.* 2002;23:737–752.

Scott R, MacPherson A, Yates RW, Hussain B, Dixon J. The effect of oral selenium supplementation on human sperm motility. *Br J Urol.* 1998;82:76–80.

Sikka SC, Rajasekaran M, Hellstrom WJ. Role of oxidative stress and antioxidants in male infertility. *J Androl.* 1995;16:464–468.

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;17:530–537.

Tesarik J, Greco E, Mendoza C. Late, but not early, paternal effect on human embryo development is related to sperm DNA fragmentation. *Hum Reprod.* 2004a;19:611–615.

Tesarik J, Ubaldi F, Rienzi L, Martinez F, Iacobelli M, Mendoza C, Greco M. Caspase-dependent and -independent DNA fragmentation in Sertoli and germ cells from men with primary testicular failure: relationship with histological diagnosis. *Hum Reprod.* 2004b;19:254–261.

Tomsu M, Sharma V, Miller D. Embryo quality and IVF treatment outcomes may correlate with different sperm Comet assay parameters. *Hum Reprod.* 2002;17:1856–1862.

Twigg J, Fulton N, Gomez E, Irvine DS, Aitken RJ. Analysis of the impact of intracellular reactive oxygen species generation on the structural and functional integrity of human spermatozoa: lipid peroxidation, DNA fragmentation and effectiveness of antioxidants. *Hum Reprod.* 1998;13:1429–1436.

Vicari E, Calogero AE. Effects of treatment with carnitines in infertile patients with prostato-vesiculo-epididymitis. *Hum Reprod.* 2000;16: 2338–2342.

Vicari E, La Vignera S, Calogero AE. Antioxidant treatment with carnitines is effective in infertile patients with prostato-vesiculo-epididymitis and elevated seminal leukocyte concentration after treatment with nonsteroidal anti-inflammatory compounds. *Fertil Steril.* 2002; 78:1203–1208.

World Health Organization (WHO). *WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction.* 4th ed. Cambridge, United Kingdom: Cambridge University Press; 1999.