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

ERMANNO GRECO,* MARCELLO IACOBELLI,* LAURA RIENZI,* FILIPPO UBALDI,* SUSANNA FERRERO,* AND JAN TESARIK†‡

From the *Centre for Reproductive Medicine, European Hospital, Rome, Italy; the †MAR&Gen, Molecular Assisted Reproduction and Genetics, Granada, Spain; and the ‡Laboratoire d’Eylau, Paris, France.

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 (Grievou 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
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 epi-fluorescence microscope with a 3×100 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 t 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), 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 months. The percentage of TUNEL-positive spermatozoa was significantly lower in the antioxidant treatment group compared with the placebo group (Table 2).

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

<table>
<thead>
<tr>
<th>Group</th>
<th>Sperm Concentration (×10^6/mL)</th>
<th>Sperm Motility</th>
<th>Normal Sperm Forms</th>
<th>TUNEL-Positive Spermatozoa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antioxidant</td>
<td>18.8 ± 17.2</td>
<td>40.2 ± 25.1</td>
<td>10.5 ± 8.1</td>
<td>22.1 ± 7.7</td>
</tr>
<tr>
<td>Placebo</td>
<td>19.1 ± 17.4</td>
<td>39.9 ± 24.8</td>
<td>11.0 ± 8.2</td>
<td>22.4 ± 7.8</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>Time of Analysis</th>
<th>Sperm Concentration (×10^6/mL)</th>
<th>Sperm Motility</th>
<th>Normal Sperm Forms</th>
<th>TUNEL-Positive Spermatozoa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before treatment</td>
<td>18.8 ± 17.2†</td>
<td>40.2 ± 25.1†</td>
<td>10.5 ± 8.1†</td>
<td>22.1 ± 7.7‡</td>
</tr>
<tr>
<td>After treatment</td>
<td>27.5 ± 24.6†</td>
<td>41.6 ± 22.0†</td>
<td>8.0 ± 7.1†</td>
<td>9.1 ± 7.2‡</td>
</tr>
</tbody>
</table>

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

† P > .05.
‡ P < .001.
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
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 non-smoking 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


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