ORIGINAL ARTICLE

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Impact of antioxidant treatment on DNA fragmentation index: a double-blind placebo-controlled randomized trial

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ABSTRACT

Background: Previous reports on effect of antioxidants on sperm DNA integrity were equivocal, and there is a lack of randomized, placebo-controlled studies.

Objectives: To evaluate the efficacy of combined antioxidant treatment in subfertile men with normal reproductive hormone levels and high sperm DNA fragmentation index (DFI).

Materials and methods: This placebo-controlled, double-blind, randomized study evaluated the effects of combined antioxidant treatment in 77 men from infertile couples, with normal testosterone, LH and FSH levels and DFI ≥25%. All participants were randomly assigned to receive combined antioxidant treatment (vitamins, antioxidants and oligoelements) or placebo for six months. The primary outcome measured was DFI. Secondary outcomes were standard semen parameters. DFI and other semen parameters were, at each time point (pre-treatment, and after three and six months of treatment), compared between the treatment and the placebo group using Mann–Whitney *U*-test.

Results: Antioxidant group had higher sperm concentration after three months of treatment (median: 24.4×10^6/mL vs. 27.2×10^6/mL; $P = 0.028$) and borderline statistically significant higher concentration after six months of treatment (median: 24.4×10^6/mL vs. 33.3×10^6/mL; $P = 0.053$) compared to pre-treatment values. The DFI did not change during the 6 months of antioxidant therapy. No statistically significant difference between the antioxidant and placebo group was seen for any of the semen parameters including sperm DFI at any of the three time points.

Discussion: The increase in sperm concentration was more pronounced in the antioxidant treated group but not statistically significantly higher than among controls, perhaps due to insufficient statistical power. Previous studies have shown positive effect of antioxidant treatment on DFI and other semen parameters. However, our findings indicate that men with normal reproductive hormone levels may not be the primary target group for such therapy.

Conclusion: Six months treatment with antioxidants had no effect on sperm DFI.

INTRODUCTION

A significant proportion of men from infertile couples has elevated proportions of spermatozoa with DNA strand breaks, measured as DNA fragmentation index (DFI) (Evenson *et al.*, 1999). It is known that chance of natural pregnancy is decreased if DFI – as measured by Sperm Chromatin Structure Assay (SCSA) — is above 20%, and dramatically reduced at DFI levels above 30% (Evenson *et al.*, 1999; Spanò *et al.*, 2000; Giwercman *et al.*, 2010). Furthermore, even the results of intrauterine

insemination (IUI) and standard in vitro fertilization (IVF) seem to be compromised at increased DFI levels (Bungum *et al.*, 2007; Oleszczuk *et al.*, 2016).

Reactive oxygen species (ROS) are believed to have an important role in the aetiology of sperm DNA fragmentation. A certain amount of ROS is needed for normal sperm function, but excessive levels have a detrimental effect on sperm DNA integrity (Agarwal *et al.*, 2014). Further, higher levels of ROS are measured in semen from infertile men, compared to proven fertile (Iwasaki

& Gagnon, 1992; Aktan *et al.*, 2013). Antioxidants counteract the action of ROS. To restore oxidant–antioxidant balance, antioxidant supplementation could be a potential treatment for some cases of male infertility.

A number of studies have investigated the effect of antioxidant treatment on standard semen parameters with contradicting results (Showell *et al.*, 2014; Ahmadi *et al.*, 2016). A Cochrane analysis (Showell *et al.*, 2014) indicated that antioxidant treatment may have a beneficial effect in males with unexplained subfertility. However, few studies have sperm DNA fragmentation as an end point, and only two of them have randomized, double-blind, placebo-controlled design (Greco *et al.*, 2005; Martinez-Soto *et al.*, 2016). Furthermore, different antioxidants or combination of several antioxidants have been used in different studies. There is also a wide variance in treatment length, dosage and study population. Therefore, results cannot easily be compared.

There are several studies that indicate that vitamins, antioxidants and oligoelements, by themselves or together, improve sperm quality (Showell *et al.*, 2014; Ahmadi *et al.*, 2016; Majzoub *et al.*, 2017). In this study, the effect of a commercial dietary supplementation containing a combination of all of these compounds was explored. This supplementation has previously been reported to improve sperm DNA integrity in infertile men with asthenoteratozoospermia (Abad *et al.*, 2013) and in infertile men with varicocele (Gual-Frau *et al.*, 2015), in non-ranodmized studies. Taking into consideration that today there are very limited options for medical treatment of male subfertility, it seems important to provide evidence based data on indications for antioxidant treatment of males from infertile couples. The group of men with infertility problem and impaired semen quality is very heterogeneous, some of them having deranged reproductive hormone levels and others not presenting with any endocrinopathy. Therefore, in this double-blind, randomized, placebo-controlled study focusing on the impact of six months combined antioxidant supplementation on DFI, we selected a well-defined cohort of subfertile men with elevated levels of DFI and normal reproductive hormonal characteristics. For selection of study participants, the DFI level of 25% was selected as lower cut-off. This value was based on the above mentioned observations of decrease of in vivo as well as in vitro fertility at the SCSA DFI level of 20% and a chance of natural pregnancy close to zero if DFI exceeds 30% (Evenson *et al.*, 1999; Spanò *et al.*, 2000; Bungum *et al.*, 2007; Giwercman *et al.*, 2010; Oleszczuk *et al.*, 2016).

PATIENTS AND METHODS

Subjects

Men who had been referred for infertility – defined as at least one year of unsuccessful attempt to achieve pregnancy – to Reproductive Medicine Centre, Skåne University Hospital (SUS), Malmö, Sweden, in whom previously performed semen analysis showed DFI ≥25%, were asked to participate in the study.

The invitation was accepted by 160 of 613 men who were asked to participate. The 160 men were invited for a first screening visit at which they were given oral and written information about the study and signed the informed consent form. Inclusion started in June 2015 and ended in August 2016. The study was performed according to the Declaration of Helsinki and approved by the ll Ethical Committee of Lund University.

The inclusion criteria were as follows:

1. Age: 18–50 years,
2. Non-smoking,
3. Not being treated with antihypertensive drugs, hormones, statins, psychotropic drugs or oral cortisone for the last six months,
4. No history of anabolic steroids use,
5. Not taking antioxidant supplementation for the last six months.

Weight and height were measured, and blood and semen sample were collected and analysed for serum levels of follicle stimulating hormone (FSH), luteinizing hormone (LH) and testosterone (T) (see below).

Men who fulfilled the previously mentioned inclusion criteria 1–5 were excluded if

1. Body mass index (BMI) ≥30,
2. FSH outside the normal range of 2–8 IU/L, (g)
3. LH outside the normal range of 2–10 IU/L,
4. T < 10 nmol/L,
5. DFI <25% in a repeated semen sample.

A total of 160 men came to the first screening visit, and after exclusion and dropout, the final study group included 77 men (Fig. 1).

Study design

The study was designed as a randomized, double-blind, placebo-controlled study. Patients selected for the study were randomized for antioxidant treatment with a commercial fertility supplement containing vitamins (vitamin C 30 mg, vitamin E 5 mg and vitamin B12 0.5 μg), antioxidants (l-carnitine 750 mg,

Figure 1 Flow chart depicting inclusion process.
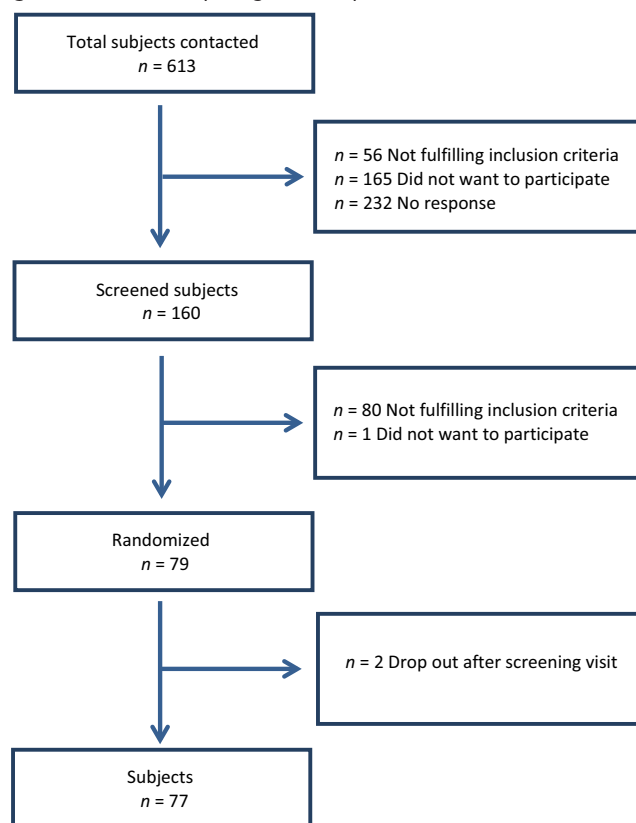
coenzyme Q10 10 mg and folic acid 100 μg) and oligoelements (zinc 5 mg and selenium 25 μg) with maltodextrin, calcium carbonate, citric acid, steviol glycoside, flavours, beta-carotene and silicon dioxide or placebo (maltodextrin, calcium carbonate, citric acid, steviol glycoside, flavours, beta-carotene and silicon dioxide) by a simple, unrestricted procedure. Both were administered orally twice per day. They were pre-packed in identical boxes and numbered according to a randomization list, by the pharmaceutical company that supplied with the products. Each participant was assigned a study number and received the corresponding pre-packed box. The allocation sequence was concealed from patients, health care providers, data collectors and researchers. Standard semen parameters and DFI were analysed before treatment and after three and six months of treatment. All patients were instructed to have an abstinence time of 2-4 days when delivering semen sample, but the actual length of abstinence period was registered in each case. At each visit possible side effects were noted. Any pregnancies during the treatment period were also reported.

Semen analysis

Semen analyses were performed according to WHO guidelines (Organization WHO, 2010). Ejaculate volume, sperm count, motility and morphology were analysed. Sperm Chromatin Structure Assay (SCSA) (Evenson, 2013) was performed to assess DFI.

Hormonal analysis

Blood samples were drawn from fasting patients between 8 and 10 a.m. Serum values of FSH and LH were measured using a one-step immunometric sandwich method with electrochemiluminiscence immunoassay. Testosterone was assessed with a two-step competitive method with electrochemiluminiscence immunoassay. All analyses were conducted at the routine clinical chemistry laboratory at SUS, Malmö.

Statistical analysis

A power calculation (http://clincalc.com/stats/samplesize.aspx) conducted prior to the study has showed that 39 patients are required in each arm in order to detect with 80% power (α = 5%) a 7% difference between the two groups in change of DFI. The power analysis was based on the assumption of mean pre-treatment DFI of 32% and a standard deviation of 11%.

There is no consensus about which level of DFI decrease is clinically relevant. A 7% decrease of DFI of 25% would bring it to a level (18%) at which it seems not to have any impact on fertility in vivo or in vitro.

Group characteristics are expressed as means (SD) and semen parameters as medians with interquartile ranges.

The distribution of the data was analysed by Shapiro–Wilk test. As most of the semen parameters deviated from normal distribution, when comparing the treatment and placebo group, a nonparametric test (Mann–Whitney U-test) was applied. Within-subject changes in sperm characteristics following three and six months therapy, with pre-treatment values as reference, were analysed by use of Wilcoxon test for paired data. Logistic regression analysis, with placebo group as reference, was performed to analyse any change in odds ratio (OR) for DFI≥25% at three and six months of treatment. Difference in proportion of men with abnormal standard semen parameters at baseline was tested by means of Fisher's exact test. Statistical calculations were performed using SPSS version 22 (SPSS, Chicago, IL, USA). A P-value <0.05 was considered as statistically significant.

RESULTS

Subjects

Seventy-nine men were randomized, and after screening visit, two men announced that they wanted to discontinue participation due to inability to stick to the schedule for delivery of semen samples after three and six months of treatment. Of the remaining 77 men, 37 were randomized to antioxidant treatment and 40 to placebo. Two men missed three months visit, and two other men missed six months visit. The reason in all four cases was that the subjects, due to lack of time, missed the ±2 days time window for the visit.

The two groups did not differ as considers age, BMI or levels of FSH, LH and testosterone (Table 1).

Semen parameters

Apart from borderline statistically significantly higher pre-treatment DFI in placebo group as compared to antioxidant treated (median: 35.5% vs. 30.0%; P = 0.053) no difference for any of the semen parameters was seen at any of the three time points (Table 2).

Placebo group had higher proportion of spermatozoa with normal form after three months of treatment (median: 2.0% vs. 4.0%; P = <0.0005) and decreased DFI after three months (median: 35.5% vs. 34.5%; P = 0.042) and six months of treatment (median: 35.5% vs. 29.5%; P = 0.005), compared to pre-treatment values. Antioxidant group had higher sperm concentration after three months of treatment (median: 24.4×10^6/mL vs. 27.2×10^6/mL; P = 0.028) and borderline statistically significant higher concentration after six months of treatment (median: 24.4×10^6/mL vs. 33.3×10^6/mL; P = 0.053) compared to pre-treatment values. On the other hand, semen volume was decreased after six months of treatment in the antioxidant group (median: 3.84 mL vs. 3.35 mL; P = 0.026). No statistically significant change was seen in DFI, total sperm count, total motility or forward motility.

As indicated in Fig. 2, the effect of combined antioxidant treatment on the DFI was independent of the pre-treatment DFI level. This result was confirmed by logistic regression analysis showing no statistically significant change in OR for DFI ≥25% after three months of antioxidant treatment (OR = 0.84; 95% CI: 0.30; 2.40) or after six months of antioxidant treatment (OR = 2.4; 95% CI: 0.84; 6.9).

Table 1 Clinical background parameters before treatment. Results are expressed as mean ± SD or in per cent

	Antioxidant group	Placebo group	P-value
Age (years)	38.0 ± 5.2	37.3 ± 4.9	0.58
BMI (kg/m^2)	24.5 ± 2.8	25.0 ± 2.7	0.44
Testosterone (nmol/L)	17.3 ± 4.2	17.0 ± 5.1	0.48
FSH (IU/L)	4.7 ± 2.1	4.4 ± 1.2	0.84
LH (IU/L)	4.9 ± 1.2	4.9 ± 1.8	0.76
Percentage with abnormal pre-treatment sperm parameters according to WHO	35	34	1.0

ANDROLOGY

Table 2 Sperm parameters before treatment, after three months and after six months of treatment with antioxidants or with placebo. Results are expressed as median (Q1–Q3)

Sperm parameters	Pre-treatment	3 months	6 months
DFI (%)			
Antioxidant group	30.0 (27.0–41.5)	30.0 (25.0–38.5)	34.0 (26.3–41.8)
Placebo group	35.5 (30.3–44.8)	34.5 (25.8–42.0)	29.5 (22.5–41.3)
P-value	0.05	0.27	0.18
Sperm concentration ($\times 10^6$/mL)			
Antioxidant group	24.4 (7.8–37.8)	27.2 (11.0–61.1)	33.3 (11.8–77.0)
Placebo group	32.2 (11.5–65.5)	30.5 (15.8–87.6)	38.8 (13.2–78.0)
P-value	0.17	0.34	0.92
Total sperm count ($\times 10^6$)			
Antioxidant group	84.2 (23.4–152.5)	74.5 (34.4–160.6)	98.1 (34.9–224.5)
Placebo group	121.0 (44.7–177.9)	102.6 (35.8–196.6)	95.1 (40.3–232.8)
P-value	0.20	0.35	0.82
Volume (mL)			
Antioxidant group	3.7 (2.6–5.0)	3.1 (2.1–4.6)	3.0 (2.0–4.1)
Placebo group	3.2 (2.5–5.1)	3.4 (2.4–4.6)	3.5 (2.2–4.6)
P-value	0.74	0.52	0.35
Total motility (%)			
Antioxidant group	59.0 (49.0–72.0)	65.0 (50.0–73.5)	61.5 (46.5–69.5)
Placebo group	62.0 (44.3–73.8)	59.5 (43.0–77.3)	60.0 (47.0–73.0)
P-value	0.94	0.51	0.82
Forward motility (%)			
Antioxidant group	43.0 (19.5–56.0)	38.0 (23.5–56.0)	36.0 (25.8–48.8)
Placebo group	40.0 (24.5–53.5)	42.0 (19.0–56.5)	40.0 (25.0–56.0)
P-value	0.93	0.90	0.41
Normal sperm forms (%)			
Antioxidant group	2.5 (1.0–5.0)	3.5 (1.0–9.5)	3.0 (1.0–6.8)
Placebo group	2.0 (1.0–6.0)	4.0 (2.0–9.0)	4.0 (1.0–8.0)
P-value	0.80	0.23	0.46
Abstinence time (days)			
Antioxidant group	2 (2–3)	2 (2–3)	2 (2–3)
Placebo group	2 (2–3)	2 (2–3)	2 (2–3)
P-value	0.80	0.23	0.46

Fertility

Seven pregnancies were achieved during study period. Three of them occurred in couples where the male underwent therapy with antioxidants, two spontaneous pregnancies and one achieved using IVF. Four spontaneous pregnancies were achieved in couples where the male underwent placebo therapy.

Safety

Generally, treatment was tolerated well. Two men reported gastrointestinal problem during treatment, one in antioxidant group and one in placebo group. One man in the antioxidant group was diagnosed with hypertension four months after study entry and commenced antihypertensive treatment.

DISCUSSION

The main finding of current study was that in subfertile men with high DFI and normal levels of LH, FSH and testosterone, as compared to the placebo group, no statistically significant effect of six months of combined antioxidant treatment on sperm DFI or any of the standard sperm parameters was observed.
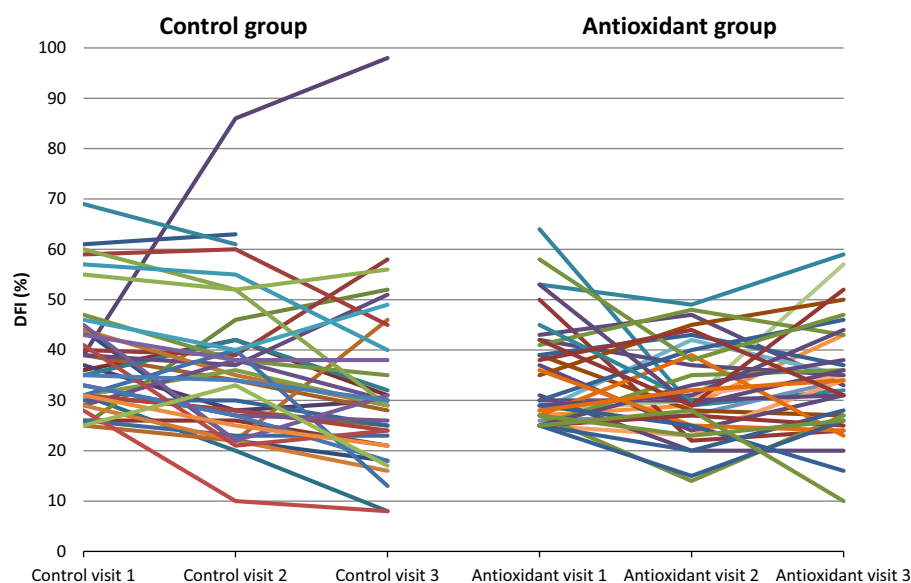

Figure 2 Individual DFI levels for each subject, at baseline (visit 1) and at three (visit 2) and six months (visit 3) of placebo or antioxidant treatment. [Colour figure can be viewed at wileyonlinelibrary.com]

At the baseline, the placebo group – as compared to the antioxidant group - presented with borderline statistically significantly higher DFI. However, although – during the 6 months of treatment – median DFI decreased slightly in the former and increased in the latter group, the differences at 3 and 6 months were not statistically significant and the discrepancy in baseline median DFI levels did not have any impact on the final conclusion of the study. A more distinctly lower DFI in the treatment group as compared to the placebo group should be expected if the antioxidant treatment caused a decrease in the level of sperm DNA fragmentation.

A number of studies have tried to elucidate the effect of antioxidant supplementation on different semen parameters. In a review by Showell *et al.* (2014), they concluded that the evidence for antioxidant supplementation was inconclusive, but clinicians should consider recommending antioxidants for subfertile men being part of an assisted reproductive programme. Another review by Agarwal *et al.* (2014) indicated that generally combined antioxidants seemed to be more beneficial than single antioxidant treatment. Even though it is well known that ROS has a detrimental effect on sperm DNA, there are only two randomized, controlled trials that have DFI as an end point. A reduction in DFI was seen after two months of daily treatment with 1 g vitamin C and 1 g vitamin E (Greco *et al.*, 2005). In another study, a reduction in DFI in subfertile men undergoing evaluation for infertility was seen after ten weeks of docosahexaenoic acid (DHA) supplementation (Martinez-Soto *et al.*, 2016). Unlike our study, Greco *et al.* (2005) and Martinez-Soto *et al.* (2016) did not exclude patients with abnormal reproductive hormone levels or those with DFI in a range where the chance of spontaneous pregnancy is not seriously reduced. Due to intraindividual variations, we only included men with DFI≥25% in two semen samples. This study also had longer treatment duration of six months, to ensure that a whole sperm regeneration cycle is within treatment period and to see if any initial changes in semen parameters persist after six months.

Varying levels of ROS is found in seminal plasma, and it originates from both endogenous and exogenous sources. Oxidative stress occurs when the amount of ROS overwhelms the natural antioxidant defence. This is known to cause damage on the spermatozoa, by damaging its DNA and by lipid peroxidation, affecting sperm membrane function (Agarwal *et al.*, 2014). With Intracytoplasmic Sperm Injection (ICSI), there is an efficient way of helping a couple achieve pregnancy even with poor semen quality. Despite great progress made in the field of assisted reproductive techniques (ART), the focus of treating infertility should primarily be on helping the couple achieve a natural pregnancy, if possible. Beyond economic reasons, there are also concerns of ART bypasses the biological mechanisms of sperm selection.

In cases where it is likely that oxidative stress could be a part of the aetiology of the male subfertility, antioxidant supplementation has been proposed as a possible solution, both to increase the chance for natural conception and also to increase the success rate of assisted reproduction. The aetiology of increased ROS levels in the male urogenital system is quite heterogeneous (Ko *et al.*, 2014). It is, therefore, plausible that not all men with high DFI may respond equally to antioxidant treatment.

In this study, we selected men with DFI at a level where impairment of in vivo and in vitro fertility could be expected and

no present deviations in levels of reproductive hormones. Also smokers and obese men were excluded in this study. It might be that these groups would benefit the most from antioxidant supplementation, as these are the conditions linked with increased oxidative stress (Tremellen, 2008). The same might be true for men with increased DFI due to endocrine disturbances, for example, low testosterone levels. Varicococele is another condition associated with increased levels of oxidative stress and elevated levels of DNA fragmentation in spermatozoa (Agarwal *et al.*, 2012). In a study by Gual-Frau *et al.* (2015), 20 infertile men with asthenoteratozoospermia and varicococele were treated with the same combined antioxidant treatment and dosage used in this study, for three months. After treatment, the relative reduction in sperm DNA fragmentation was statistically significant relative to pre-treatment values. Although the study was not placebo-controlled, the results could indicate that this stratified group of infertile men could be a possible target group where antioxidant treatment could be beneficial.

Following 3 months antioxidant treatment a slight, but statistically significant increase in sperm concentration was observed. This increase was only borderline significant after 6 months of treatment, and total sperm count remained unchanged during the whole study period. Therefore, the risk of type 2 error needs to be considered.

In conclusion, in this double-blind, randomized, placebo-controlled study, we did not find any improvement in either sperm DNA fragmentation or standard semen parameters, after six months of combined antioxidant treatment of men with normal levels of reproductive hormones and high DFI. The outcome of use of antioxidants in other subgroups of subfertile men needs to be tested in future studies to provide scientific evidence for what by some clinicians is considered to be a useful treatment.

CONFLICT OF INTEREST

The authors have no other conflict of interests relating to this study.

AUTHORS' CONTRIBUTIONS

AG and KO designed the study, AS and IL performed the clinical research and collected data, AS and AG analysed the data, AS wrote the paper, AG, KO and IL critically revised and all authors approved the final version of the manuscript.

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