

Article

Antioxidants to reduce sperm DNA fragmentation: an unexpected adverse effect



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Abstract

Reactive oxygen species (ROS) have a negative impact on sperm DNA, leading to the formation of oxidative products such as 8-oxo-7,8-dihydroxyguanosine. This compound causes fragmentation and, thus, has a mutagenic effect. Patient treatment with oral antioxidant vitamins is, therefore, standard practice for male infertility, in an attempt to decrease formation of ROS and improve fertility. In this study, the DNA fragmentation index and the degree of sperm decondensation were measured using the sperm chromatin structure assay before and after 90 days treatment with antioxidant vitamins associated with zinc and selenium. Antioxidant treatment led to a decrease in sperm DNA fragmentation (-19.1% , $P < 0.0004$), suggesting that at least part of the decay was linked to ROS. However, it also led to an unexpected negative effect: an increase in sperm decondensation with the same order of magnitude ($+22.8\%$, $P < 0.0009$). The opening of interchain disulphide bridges in protamines may explain this aspect, as antioxidant vitamins, especially vitamin C, are able to open the cystin net, thus interfering with paternal gene activity during preimplantation development. This observation might explain the discrepancy observed concerning the role of these antioxidant treatments in improving male fertility.

Keywords: antioxidants, reactive oxygen species, sperm condensation, sperm DNA fragmentation

Introduction

Sperm quality is one of the most important areas of concern in treatments involving assisted reproductive technology. Although progress has been made via the use of intracytoplasmic sperm injection (ICSI), the question of sperm DNA quality remains of major importance. Human spermatozoa have a high content of polyunsaturated fatty acids and a limited capacity for DNA repair. This renders them extremely sensitive to attack by reactive oxygen species (ROS). Sperm DNA fragmentation as an indicator of quality has recently become an area of considerable interest, and a variety of methods have been developed in order to evaluate paternal DNA integrity. This assessment of

sperm quality is independent of all basic semen parameters including sperm morphology, concentration and motility. The different technologies that have been applied have reached the same conclusion: sperm DNA fragmentation leads to reduced fertility (Kodama *et al.*, 1997; Henkel *et al.*, 2004), particularly for artificial insemination with partner's spermatozoa and in routine IVF (Evenson *et al.*, 2002). Results suggest that ICSI may sometimes, but not always, improve embryonic developmental potential for sperm samples that display this type of DNA fragmentation (Lopes *et al.*, 1998a).

Reactive oxygen species have a seriously deleterious effect on sperm DNA. They lead to formation of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo), the major oxidative product of sperm DNA. This compound causes fragmentation (Lopes *et al.*, 1998b), and therefore has a mutagenic effect. During the process of fertilization, factors in the oocyte cytoplasm should theoretically repair any decays in the integrity of sperm DNA (Hamatani *et al.*, 2004; Zeng *et al.*, 2004). The DNA repair capacity is decreased with age. In the absence of repair, such DNA lesions have profound effects on cell viability and have the potential of oncogenic transformation: transmission of altered DNA is associated with an increase in childhood cancer (Boiteux and Radicella, 1999; Zenzes, 2000; Cline and Hanawalt, 2005). It has therefore been tempting to try to reduce or avoid the generation of DNA fragmentation related to free radical exposure. Numerous controversial studies have evaluated the efficacy of antioxidants in the treatment of male infertility (Agarwal *et al.*, 2004; Greco *et al.*, 2005a,b). The majority agree that these treatments result in only partial success. This study used sperm chromatin structure to investigate the impact of antioxidant treatment, not only in terms of sperm DNA fragmentation but also with respect to sperm head decondensation. Antioxidants may have an effect by opening the interchain disulphide linkages in protamines, especially P2, leading to sperm DNA compaction (Bedford and Calvin, 1974).

Materials and methods

The experiments were performed in two centres of assisted reproductive biology, one near Lyon, France (Centre de FIV de la clinique du val d'Ouest) and one in Paris (Clinique le Muette, laboratoire d'Eylau). Patients who had at least two previous failures of IVF or ICSI were enrolled in the study. A sperm chromatin structure assay (SCSA; Evenson *et al.*, 2002) was performed on sperm samples. After acidic denaturation, cells were dyed with acridine orange. Then sperm samples were analysed in a flow cytometer (Beckman Coulter Epics XL-MCL). SCSA software allows the separation and analysis of spermatozoa with red fluorescence and those exhibiting green fluorescence. Lack of appropriate maturation or anomalies in compaction result in an increase

in the amount of DNA staining (green fluorescence). Red fluorescence is a marker of single-stranded DNA decay. A minimum concentration of 1×10^6 spermatozoa/ml is required for the assay. In addition, a strict minimal abstinence of 3–5 days and a negative semen culture were required because these factors could increase DNA fragmentation. If either the DNA fragmentation index (DFI) or the degree of sperm decondensation was $> 15\%$, the patients were prescribed a daily oral antioxidant treatment consisting of vitamins C and E (400 mg each), β -carotene (18 mg), zinc (500 μ mol) and selenium (1 μ mol): this is the classic treatment given by andrologists (see Agarwal *et al.*, 2004). The value of 15% for both parameters corresponds to half of the threshold at which fertility is impaired: 30% for DFI (Evenson *et al.*, 2002) and 28% for decondensation of spermatozoa (authors' own observation). The duration of treatment was 90 days, in order to provide protection during the complete cycle of spermatogenesis. At the end of the treatment period, the DFI and the degree of sperm decondensation were checked and recorded. Statistical analysis was performed using Student's *t*-test and the Wilcoxon test.

Results

Fifty-eight patients fulfilled the enrolment requirements of the study. When these patients were analysed, a significant decrease (-19.1%) in DFI was observed, from 32.4% ($\sigma = 12.2$) to 26.2% ($\sigma = 10.8$), ($P < 0.0004$ for Student test, $P < 0.001$ for Wilcoxon test) (Table 1, Figures 1, 2).

In contrast, a significant increase in sperm decondensation ($+22.9\%$) was observed from 17.5% ($\sigma = 12.2$) to 21.5% ($\sigma = 13.6$) ($P < 0.0009$ for Student's *t*-test, $P < 0.001$ for Wilcoxon

Table 1. Difference in DNA fragmentation index (DFI) and degree of high DNA stainability (HDS) in 58 patients following treatment with antioxidants.

	Before treatment	After treatment	P-value
DFI (%)	32.4	26.2	0.0004
HDS (%)	17.5	21.5	0.0009

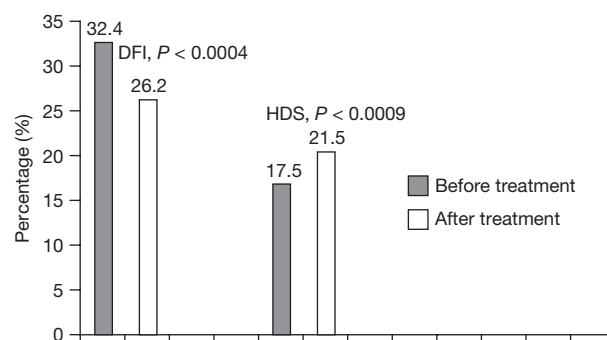


Figure 1. Decrease in DNA fragmentation index (DFI) and increase in sperm decondensation in 58 patients after treatment with antioxidants. Grey: before treatment; white: after treatment. DFI = DNA fragmentation index, HDS = degree of decondensation of spermatozoa.

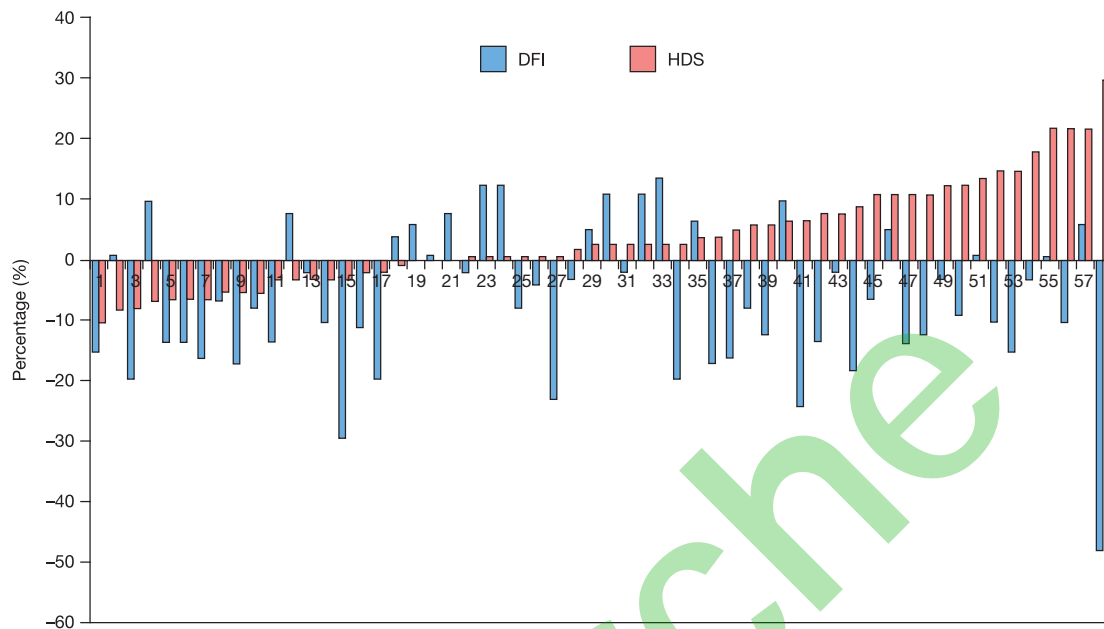


Figure 2. Individual variations in DNA fragmentation index and sperm decondensation in spermatozoa after 90 days treatment with antioxidant. DFI = DNA fragmentation index, HDS = high DNA stainability. Patients are ranked according to degree of HDS. – Decrease

test) (Table 1, Figures 1, 2).

Discussion

Even under physiological conditions, gamete and zygote genomes are continuously submitted to attack. In human IVF and ICSI, *in-vitro* manipulations undoubtedly increase these assaults, especially in relation to ROS exposure. Patient treatment with oral antioxidant vitamins is a standard practice in male infertility, in an attempt to improve sperm quality. The rationale behind this is to decrease the formation of ROS by neutralizing superoxide anions ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl (OH^\bullet) radicals, thus decreasing the potential decay of membrane lipid and DNA. *In vitro*, vitamin C and alpha-tocopherol may have ambiguous effects, intensifying the DNA decay even if the production of ROS is reduced (Donnelly *et al.*, 1999). This confirms that DNA strand breaks are not solely induced by ROS. A careful analysis of the various dosages described in the literature shows that, *in vivo*, these treatments are either effective or useless, irrespective of a significant decrease in ROS generation observed in sperm and seminal plasma.

However, several of these antioxidant treatments improve the fertilization rates for ICSI, even in the absence of significant improvements observed for sperm parameters (Lewin and Lavon, 1997). Intake of oral vitamins does reduce DNA fragmentation, but it also increases sperm decondensation by more than 25%. This is definitely a negative effect, as the nuclear structure of sperm chromatin influences the initiation and regulation of paternal gene activity during preimplantation

development (Haaf and Ward, 1995). An unwanted high degree of sperm decondensation can result in asynchronous chromosome condensation, and may lead to cytoplasmic fragments in the embryo. In the authors' clinics, no pregnancy has been observed following IVF or ICSI when sperm decondensation is $> 28\%$. Contrary to the situation concerning DNA breaks (Greco *et al.*, 2005a,b), ICSI does not improve the results for high decondensation. The negative effect on chromatin packaging seems to originate from the high reducing potential linked to these reducing agents. In a small cohort of patients, the same observations (chromatin decondensation and reduction in DFI) were observed when a modified superoxide dismutase was given orally alone instead of vitamins. In a small cohort of 12 patients the same variations were observed with an even greater increase in sperm decondensation (data not shown). This secondary effect is probably due to disulphide bond reduction in protamines. Due to its high redox potential, vitamin C can reduce cystine to two cysteine moieties, and thus open the interchain disulphide bridges.

It is difficult to compare this data with the situation *in vivo* (Moustafa *et al.*, 2004; Hammadeh *et al.*, 2006; Appasamy *et al.*, 2007). Here again the results are controversial: it is obvious that DNA damage is induced by oxidative assaults, but the physiological antioxidant situation in the seminal plasma does not seem to be strongly associated with either a reduction in the DNA damage or an increase in the decondensation (Hammadeh *et al.*, 2006). Moreover it has to be pointed out that the results usually originate from small cohorts of patients and that, following antioxidant treatment, the 'total antioxidant status' reached *in vivo* is the most important factor and this is likely to be different from the

situation observed *in vitro*.

In conclusion, care must be taken in the use of antioxidant treatment for improving sperm quality, especially considering the decay in the integrity of DNA. The quality of DNA packaging seems to be equally important. Antioxidants should not be recommended in men whose semen samples show a degree of decondensation over a threshold of 20% in order to avoid reaching the critical value of 28%. Below this value, the risk:benefit ratio is in favour of antioxidant treatment. This observation might ultimately explain the wide discrepancy observed in the scientific literature concerning the role of long-term antioxidant treatments in improving fertility (Agarwal *et al.*, 2004), especially if individual variations between patients are considered.

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