Article

Improvement in sperm DNA quality using an oral antioxidant therapy

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Abstract

Oxidative stress is now recognized as a common pathology that affects up to half of all infertile men. One of the principal mechanisms by which oxidative stress produces infertility is by damage to sperm DNA, either through direct oxidation of the DNA by reactive oxygen species (ROS) or by the initiation of apoptosis. The objective of this study was to determine if an oral antioxidant/mineral supplement could improve sperm DNA integrity in men with known oxidative stress. A total of 50 infertile men identified as exhibiting oxidative stress were administered oral antioxidant therapy for a period of 3 months. All participants were assessed at entry and exit for sperm DNA integrity with terminal deoxynucleotidyl transferase-mediated dUDP nick-end labelling, apoptosis with annexin V, protamination with chromomycin A_3 and ROS production with nitro blue tetrazolium assay. Sperm concentration, motility and morphology, together with assessment of serum male reproductive hormones (LH, FSH, testosterone, anti-Müllerian hormone), were also monitored. The principal finding that emerged from this study was that antioxidant therapy resulted in significant improvements in sperm DNA integrity ($P = 0.002$) and protamine packaging ($P < 0.001$), accompanied by a reduction in seminal ROS production ($P = 0.027$) and apoptosis ($P = 0.004$). No significant changes in routine sperm parameters (concentration, motility, morphology) or male reproductive hormones were observed.

Keywords: *antioxidant, apoptosis, protamine, reactive oxygen species, sperm DNA*

Introduction

Male factor infertility is recognized to account for half of all cases of infertility, with one in 20 men in the general population exhibiting some form of defect in sperm quality (McLachlan and de Krester, 2001). While male fertility has traditionally been defined by sperm density, motility and morphology, more recently sperm DNA quality has been recognized as one of the most important determinants of male reproductive potential (Lewis and Aitken, 2005; Ozmen, 2007; Tarozzi, 2007). Normal embryo development beyond the cleavage stage is dependent in part on the quality of paternal DNA contained within the fertilizing spermatozoon (Seli *et al*., 2004; Benchaib *et al*., 2007). Elevated levels of sperm DNA damage are associated with increased time to natural conception (Spano *et al*., 2000; Loft *et al*., 2003), decreased intrauterine insemination and IVF/intracytoplasmic sperm injection (ICSI) pregnancy rates (Larson *et al*., 2000; Benchaib *et al*., 2003; Bengum *et al*., 2004; Virro *et al*., 2004; Borini *et al*., 2006), increased miscarriage risk (Virro *et al*., 2004; Borini *et al*., 2006; Benchaib *et al*., 2007) and possibly even increases in childhood cancer (Lewis and Aitken, 2005). As at least 15% of men in infertile relationships have compromised sperm DNA integrity (Benchaib *et al*., 2007), the development of new treatments to improve sperm DNA quality is of major clinical importance.

While the exact causes of sperm DNA damage have not been fully elucidated, several interrelated mechanisms have been suggested (Tesarik, 2006; Aitken and De Iuliis, 2007; Ozmen *et al*., 2007; Tarozzi, 2007). Firstly, the most widely accepted cause for sperm DNA fragmentation is the presence of oxidative stress. Oxidative stress occurs when the production of reactive

oxygen species (ROS) by spermatozoa and seminal leukocytes exceeds the semen's intrinsic antioxidant capacity, thereby leading to unopposed free radical attack on spermatozoa (Tremellen, 2008). This link between oxidative stress and sperm DNA damage is irrefutable. The generation of sperm oxidative stress *in vitro*, either through the direct application of hydrogen peroxide (Aitken *et al*., 1998) or by stimulation of the spermatozoon's own intrinsic production of free radicals (Twigg *et al*., 1998), has been reported to significantly increase the levels of DNA damage within spermatozoa.

Oxidative damage to spermatozoa is very common, affecting between 30% and 80% of infertile men, and is due to several predisposing factors (Tremellen, 2008). Firstly, infertile men's semen often contains more morphologically abnormal, immature spermatozoa that have an increased capacity to produce ROS compared with mature spermatozoa (Gomez *et al*., 1996). Secondly, infertile men often have reduced antioxidant capacity within their seminal plasma and spermatozoa (Fujii *et al*., 2003; Tremellen, 2008), thereby exposing the spermatozoa to a greater risk of oxidative attack. Finally, inflammation within the male genital tract is present in a significant proportion of infertile men (Henkel *et al*., 2003). Neutrophils, the dominant leukocyte contained within semen, are renowned for their capacity to generate significant amounts of ROS when activated, producing an oxidative burst that can then result in oxidative damage to sperm DNA (Saleh *et al*., 2002; Henkel *et al*., 2005).

Defective protamine packaging of sperm DNA may also make infertile men's spermatozoa more susceptible to oxidative attack (Ozmen *et al*., 2007). During normal spermatogenesis, 85% of nuclear histones are replaced with protamines that allow tight packaging of the sperm DNA, protecting it from oxidative attack. Approximately 15% of infertile men have been reported to exhibit deficient protamine packaging (Carrell and Liu, 2001), making these men's spermatozoa more vulnerable to oxidative DNA damage (Aoki *et al*., 2005; Torregrosa *et al*., 2006). Furthermore, the replacement of histones with protamines requires transient breaks in sperm DNA to be produced by the enzyme topoisomerase II. These physiological DNA breaks are then usually resealed by topoisomerase II at the spermatid stage of spermatogenesis. However, it has been proposed that altered topoisomerase II activity may lead to defective repair and residual DNA fragmentation (Tarozzi, 2007).

The oxidation of protamines during the late stages of spermatogenesis also plays a role in determining sperm DNA's susceptibility to oxidative attack. As spermatozoa pass from the testis to the epididymis, protamine thiol groups become oxidized, enhancing the stability of the sperm DNA by forming strong intra- and intermolecular disulphide cross-links. In the mouse, the seleno-protein phospholipid hydrogen peroxide glutathione peroxidase (PHGPx) is responsible for the oxidation of free thiols and the creation of disulphide bridges (Conrad *et al*., 2005). Knock-out mice with the PHGPx enzyme silenced have significantly higher concentrations of free thiols, impaired chromatin disulphide bond formation and defective chromatin condensation (Conrad *et al*., 2005). Interestingly, spermatozoa from oligoasthenoteratospermic (OAT) men are characterized by biphasic thiol distribution patterns, reflecting both incomplete and excessive thiol oxidation (Ramos *et al*., 2008). In this particular study, lower concentrations of free thiol groups, consistent with a state of oxidative stress, were associated with an increase in sperm DNA fragmentation, accessed by the TdT (terminal deoxynucleotidyl transferase)-mediated dUTP nick-end labelling [TUNEL] assay.

The final mechanism responsible for sperm DNA fragmentation is abortive apoptosis (Sakkas *et al*., 2003). Apoptosis plays two principal roles in spermatogenesis. Firstly, it is essential in limiting the population of germ cells to a number that can be adequately supported by Sertoli cells, thus ensuring normal spermatogenesis. Secondly, apoptosis is proposed to be responsible for the selective depletion of abnormal germ cells (Sakkas *et al*., 2003; Tarozzi, 2007). However, even when good-quality spermatozoa are exposed to oxidative stress, apoptotic destruction is often triggered (Moustafa *et al*., 2004). If apoptosis fails to completely destroy these damaged cells (abortive apoptosis), endonuclease-mediated digestion of the sperm DNA will occur within these still viable spermatozoa. Therefore, oxidative damage to sperm DNA is the net effect of direct oxidative damage to the purine and pyrimidine bases of the sperm DNA, in tandem with apoptotic endonuclease digestion.

The Menevit® nutraceutical is an antioxidant preparation that has recently been shown in a placebo-controlled randomized study to improve pregnancy outcomes when used in conjunction with IVF-ICSI treatment (Tremellen *et al*., 2007). The seven different components of Menevit® (**Table 1**) were identified as suitable for promoting sperm DNA health because of their capacity to impede oxidative attack through three overlapping mechanisms. Firstly, vitamins C and E, selenium, garlic and lycopene have direct antioxidant effects (Heber and Lu, 2002; Saravanan and Prakash, 2004; Valko *et al*., 2004), assisting in the neutralization of ROS already produced by spermatozoa or seminal leukocytes. Secondly, lycopene (Heber and Lu, 2002) and garlic (Hodge *et al*., 2002) have been shown to have potent anti-inflammatory activity, thereby potentially resulting in a reduction in seminal leukocyte ROS production. Finally, zinc, folate and selenium are known to play a vital role in sperm DNA synthesis and protamine packaging (Kvist *et al*., 1987; Evenson *et al*., 1993; Pfeifer *et al*., 2001; Brewer *et al*., 2002;), possibly protecting sperm DNA from oxidative stress. The aim of the current Assessment of DNA After Menevit® (ADAM) study was to determine if the Menevit® antioxidant can alter oxidative attack, protamine packaging, sperm DNA integrity and the production of male reproductive hormones.

Table 1. Contents of the Menevit® antioxidant.

Component	*Amount per unit*
Lycopene	6 mg
Vitamin E	400 IU
Vitamin C	100 mg
Zinc	25 mg
Selenium	26 µg
Folate	500 µg
Garlic oil	333 µg (equivalent 1 g garlic)

Materials and methods

Subjects and study design

Participants in the ADAM study were recruited from men undergoing infertility assessment at an academic affiliated assisted reproductive technology unit (Repromed, Dulwich, South Australia). To be eligible for involvement, study participants were required to meet two inclusion criteria. Firstly, the entry semen analysis had to exhibit significant oxidative stress, as assessed by the nitroblue tetrazolium (NBT) assay. Levels of seminal ROS production exceeding the 75th percentile for fertile donors (19 µg formazan/10^7 spermatozoa) were considered as sufficient evidence for oxidative stress. A previous pilot study of 12 fertile donors and 25 randomly selected infertile men had found that 68% of infertile men had a seminal ROS score exceeding the 75th percentile for fertile donors (O Tunc *et al.*, unpublished observation). Secondly, participants were required to have a minimum sperm concentration of 1×10^6/ml, as this was the minimum needed to conduct the various sperm DNA quality assays.

A total of 56 men with probable oxidative stress, determined by poor motility, high abnormal sperm morphology and altered semen viscosity on routine analysis (Tremellen, 2008) were screened to identify the target sample of 50 men with confirmed oxidative stress. Three men were excluded from the study due to low sperm count and three because of inadequate levels of oxidative stress on NBT assessment. The average age of participants was 39 ± 5.8 years (range 26–53 years) with an average duration of infertility of 2.5 ± 0.6 years.

Those participants who tested positive for oxidative stress were asked to take one capsule of Menevit® (Bayer Australia Ltd, Sydney, Australia) per day for a period of 3 months and to provide a semen and serum sample both at entry and exit. Four men withdrew from the study before completing their 3 months of antioxidant treatment due to a lack of continuing interest. One man withdrew as he believed the treatment aggravated his symptoms of irritable bowel syndrome. The study was prospectively approved by the Human Research and Ethics Committee, Women's and Children's Hospital (approval REC 1942/4/10), with all participants giving written informed consent for their involvement.

Sample collection and preparation

Semen samples were produced by masturbation after a period of 3–5 days abstinence and then analysed for sperm count, motility and morphology as per World Health Organization (1999) guidelines. After liquefaction, 400 µl of semen was suspended in 2 ml of Dulbecco's phosphate-buffered saline (PBS) (JRH Biosciences, Kansas, USA) and washed twice and pelleted by centrifugation at 300 *g* for 5 min. The washed semen was then resuspended in 400 µl PBS and used in either the NBT and annexin V assays or smeared on poly-L-lysine coated slides for fixation and later TUNEL and DNA protamination (chromomycin A_3, CMA_3) analysis.

All serum samples were obtained by venepuncture between the hours of 09:00 and 11:00, and frozen at –70°C until the relevant reproductive hormones (LH, FSH, testosterone, and anti-Müllerian hormone [AMH]) were measured.

Assessment of sperm DNA fragmentation (TUNEL)

Sperm DNA fragmentation was detected by the *In situ* Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany), which is based on the labelling of DNA strand breaks (TUNEL technology). This assay was performed using a modification of the microscopic TUNEL technique (Gandini *et al.*, 2000; Benchaib *et al.*, 2003). Briefly, spermatozoa were smeared on poly-L-lysine coated slides, air dried and fixed with 3:1 methanol/glacial acetic acid fixative. The spermatozoa were then permeabilized with 0.1% Triton X-100/0.1% sodium citrate and washed with PBS before being incubated with fluorescein isothiocyanate (FITC)-labelled terminal deoxyribonucleotidyl transferase, followed by fluorescein labelling with propidium iodide. The smear was rinsed in PBS buffer and mounted in a 1:1 mixture of ProLong® Gold antifade reagent (Invitrogen Molecular Probes, OR, USA) and glycerol. Stained cells were quantified on an Olympus BX51 fluorescence microscope, with a minimum of 300 spermatozoa per slide being assessed using image analysis software (MacProbe V 4.3, Perceptive Scientific Instruments, League, TX, USA). The percentage of sperm DNA fragmentation was calculated as the number of TUNEL-positive nuclei (FITC-labelled, green) per total number of sperm nuclei (propidium iodide, red). For a positive control, sperm cells were incubated with 3 IU/µl DNase prior to incubation with the TUNEL reagents, and for a negative control, the terminal transferase was omitted from the reaction.

Assessment of sperm apoptosis (annexin V)

Apoptotic sperm cells were identified using the annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich, St. Louis, USA). During the early stages of apoptosis, phosphatidyl serine (PS) becomes translocated onto the outer leaflet of the sperm membrane. Since annexin V antibody binds specifically to externalized PS, the presence of annexin V fluorescence is a marker of sperm apoptosis. The procedure was conducted according to the protocol recommended by the manufacturer. The PBS washed spermatozoa were diluted to a concentration of 1×10^6 cell/ml with the relevant amount of binding buffer (100 mmol/l HEPES/NaOH buffer), before 1 µl annexin V and 0.5 µl propidium iodide were added to the cell suspension. After incubation at room temperature for 10 min under dark conditions, the suspension was centrifuged at 400 *g* for 5 min and the pellet was mounted on poly-L-lysine coated slides. At least 300 individual spermatozoa were then examined by fluorescence microscopy, as previously outlined in the TUNEL protocol. Spermatozoa that stained positive for annexin V (green), but that excluded the red propidium iodide dye were considered to be alive but in the early stages of apoptosis.

Assessment of sperm protamination (CMA_3)

Quantification of sperm DNA protamination was made using the CMA_3 fluorochrome that indirectly demonstrates a reduced presence of protamine in the sperm nucleus. CMA_3 is a fluorochrome specific for GC-rich sequences in DNA and competes with protamines for the same binding sites in the minor groove of DNA. PBS washed spermatozoa were smeared on

slides and then fixed in (3:1) methanol/glacial acetic acid at 4°C for 5 min, before being treated for 20 min with 100 µl of CMA_3 solution (0.25 mg/ml in McIlvaine buffer, pH 7, containing 10 mmol/l $MgCl_2$; Sigma-Aldrich), as previously described by Manicardi *et al.* (1995). The slides were then rinsed in buffer and mounted in a 1:1 mixture of ProLong® Gold antifade reagent and glycerol. Microscopic analysis of at least 300 spermatozoa was performed on an Olympus BX51 fluorescence microscope with the appropriate filter (460 nm). Spermatozoa were evaluated by distinguishing those spermatozoa with bright yellow staining (CMA_3 positive) from those with dull yellow staining (CMA_3 negative). The degree of correct protamination for each sample was then determined by subtracting the CMA_3 positivity from 100%.

Measurement of ROS production

A modified colorimetric NBT test was used to evaluate ROS production of both leukocytes and sperm cells within semen (Mercardo-Pichardo *et al.*, 1981; Esfandiari *et al.*, 2003; Tunc *et al.*, 2008). After washing in PBS, spermatozoa were resuspended in 200 µl of PBS and incubated with NBT reagent (0.01% NBT in PBS, Sigma-Aldrich) at 37°C for 45 min. Following incubation the samples were washed and centrifuged at 500 *g* for 10 min in PBS to remove all residual NBT solution, leaving only a cell pellet. Formazan, a blue water-insoluble crystal produced from the yellow water-soluble tetrazolium salt by the action of cellular superoxide anions, is then deposited inside the spermatozoa and leukocytes. The amount of formazan crystal present within a cell is closely related to its production of free radicals (Esfandiari *et al.*, 2003). In order to quantify the formazan product, the intracellular formazan was solublized in 60 µl of 2 mol/l KOH and dimethyl sulphoxide (DMSO) (Sigma-Aldrich) and the colour reaction was measured spectrophotometrically on a microplate reader (Bio-Tek, USA, Model ELx800) at 630 nm, as previously reported for somatic cells (Choi *et al.*, 2006). ROS production was expressed as µg formazan per 10^7 spermatozoa, derived from a standard curve of absorbance values for known amounts of formazan substrate.

Hormone analysis

Serum samples for hormone assessment were separated within 1 h of collection and frozen at –70°C until assayed. All hormone measurements, with the exception of AMH (see below), were conducted using the automated ADVIA Centaur chemiluminescent immunoassay system obtained from Bayer Australia Ltd (Pymble, NSW, Australia). Serum AMH concentrations, a measure of Sertoli cell function (Rajpert-De Meyts *et al.*, 1999), were measured using the Immunotech high-sensitivity immuno-enzymetric assay (Beckman Coulter, Marseille, France).

Statistical analysis

Data were analysed using the statistical software SigmaStat (Systat Software Inc, CA, USA). As the majority of data was not normally distributed, the results are principally expressed as median values (inter-quartile range), with statistical analysis being performed using the non-parametric Wilcoxon signed rank test. When the data were normally distributed, results are expressed as mean ± SD and were analysed using the paired *t*-test. A *P*-value <0.05 was considered statistically significant.

Results

After 3 months of antioxidant treatment, sperm DNA integrity improved significantly, with the median sperm DNA fragmentation level dropping from 22.2% to 18.2% ($P = 0.002$; **Table 2**). Recently it has been reported that short periods of abstinence can result in spontaneous improvements in sperm DNA integrity (Bakos *et al.*, 2008). No significant difference was observed in the period of abstinence between the entry and exit samples (mean 3.8 versus 4.2 days respectively) that could account for the observed improvements in sperm DNA integrity. Furthermore, the observed improvements in sperm DNA quality were mirrored by reductions in early apoptosis (annexin V positive, propidium iodide negative; $P = 0.004$) and a significant fall in seminal ROS production ($P = 0.027$), suggesting that an antioxidant effect was primarily responsible for the observed improvement in sperm DNA integrity.

Treatment with antioxidant containing zinc and selenium for 3 months produced a small but statistically significant improvement in median levels of sperm DNA protamination (69.0% versus 73.6%, $P < 0.001$). During normal spermatogenesis, 85% of sperm histones are replaced by protamines; with impaired reproductive outcomes being observed once sperm protamination levels fall below 70% (Nasr-Esfahani *et al.*, 2004). At the commencement of the study, 26 infertile men (58%) had inadequate levels of sperm DNA protamination (<70% sperm protamine content), yet by study completion, only 14 participants (31%) exhibited protamine deficiency (<70% sperm protamine content).

Basic sperm parameters (count, motility, morphology, semen volume) did not change significantly following 3 months of antioxidant treatment (**Table 3**). All 45 trial participants had at least one defect in routine sperm quality, since only men with pre-existing male factor infertility were approached to enter the study. However, it can be seen that the average sperm parameters of the group were only moderately impaired.

Antioxidant therapy for 3 months did not appear to effect the production of any of the reproductive hormones monitored (**Table 4**). No significant changes in hormones related to Leydig cells (serum LH and testosterone) nor Sertoli cells (serum FSH, AMH) fuction were observed. Furthermore, when a subgroup analysis examining only those participants with evidence of Sertoli or Leydig cell dysfunction at trial entry (FSH >10 IU/l, testosterone <8 nmol/l) was performed, no significant differences in any reproductive hormones was observed following antioxidant therapy.

In the ADAM study, 37 participants underwent IVF treatment while on the Menevit® antioxidant, although this was not a criterion for entry to the study. The average age of the female partners was 35.7 ± 4.5 years, with an average duration of infertility 3.0 ± 1.3 years. The majority of ADAM participants underwent IVF-ICSI (36 cycles ICSI, one cycle IVF), with the observed mean fertilization rate being 68.0 ± 26.7%. Of the 37 cycles, 36 proceeded to an embryo transfer, with one transfer being cancelled due to no genetically normal embryos being available for transfer (preimplantation genetic diagnosis cycle). Sixteen of the partners achieved a clinical pregnancy, defined as one that exhibits a gestation sac on first trimester ultrasound. The vast majority of

Table 2. Sperm DNA quality before and after 3 months of antioxidant treatment.

	Entry	*Exit*	P-*value*	n
DNA fragmentation (%, TUNEL positive)	22.2 (16.5–26.6)	18.2 (13.4–23.1)	0.002	45
Early apoptosis (%, annexin V +, PI –)	27.3 (20.4–34.7)	22.5 (19.3–28.1)	0.004	45
DNA protamination (%)	69 (63.5–73.1)	73.6 (69.3–77.5)	<0.001	45
ROS production (µg formazan/10^7 spermatozoa)	66.4 (43.1–87.8)	44.4 (33.3–81.4)	0.027	45

All values are expressed as median (inter-quartile range). PI = propidium iodide; ROS = reactive oxygen species; TUNEL = TdT (terminal deoxynucleotidyl transferase)-mediated dUTP nick-end labelling. Statistical analysis was performed using the Wilcoxon signed rank test.

Table 3. Routine semen assessment before and after 3 months of antioxidant treatment.

	Entry	*Exit*
Concentration (10^6 spermatozoa/ml)[a]	36.2 ± 46.2	39.4 ± 44.7
Motility (%)[b]	36.5 ± 12.3	37.1 ± 11.5
Normal morphology (%)[b]	6.7 ± 5.0	6.71 ± 4.5
Semen volume (ml)[a]	2.94 ± 1.8	2.88 ± 1.7
Total motile spermatozoa (10^6 per ejaculate)[a]	38.4 ± 56.4	43.9 ± 64.5

All values are expressed as mean ± standard deviation.
[a]Wilcoxon signed rank test.
[b]Paired *t*-test.
There were no statistically significant differences in any of the parameters between entry and exit samples.

Table 4. Reproductive hormone concentrations before and after 3 months of antioxidant treatment.

	Entry	*Exit*
LH (IU/l)	3.93 ± 1.33	4.23 ± 1.34
FSH (IU/l)	5.58 ± 3.13	5.81 ± 3.28
Testosterone (nmol/l)	14.07 ± 4.1	14.96 ± 5.5
AMH (pmol/l)	61.8 ± 28.9	61.4 ± 28.3

All values are expressed as mean ± SD. AMH = anti-Müllerian hormone. Statistical analysis was performed using the paired *t*-test and no statistically differences were found between entry and exit values.

the scans were performed at 8 weeks' gestation. A viable clinical pregnancy is when a scan shows fetal heart pulsations. Of the 16 clinical pregnancies, 13 (81.25%) were viable on a first-trimester ultrasound. A further three couples experienced a biochemical pregnancy, giving an overall biochemical pregnancy rate of 52.8% and a clinical (ultrasound identified) pregnancy rate of 44.4% per embryo transfer. Within this viable pregnant cohort, the median sperm DNA fragmentation was reduced from 22% at entry to 13.3% at exit.

Discussion

The present data show a significant improvement in sperm DNA integrity following 3 months of antioxidant therapy. This result is consistent with the published literature. Firstly, a combination of vitamin C and E has been shown in well-conducted placebo-controlled randomized controlled trials to reduce both sperm (Greco *et al.*, 2005) and peripheral blood leukocyte DNA damage (Moller *et al.*, 2004). Non-placebo-controlled prospective studies have also shown antioxidants to improve sperm DNA integrity (Comhaire *et al.*, 2000; Ménézo *et al.*, 2007).

The presence of high levels of sperm DNA damage was not used as a criterion for entry into the ADAM study, so as to avoid any suggestion that the primary study outcome (sperm DNA quality) was biased by the regression-to-the-mean phenomenon. Baker and Kovacs (1985) had previously highlighted that, when a group of subjects are selected for extreme results, on average their results tend to normalize on re-testing –so-called a regression to the mean. This regression phenomenon is an important consideration when analysing semen parameters such as sperm count or motility, which are prone to wild intrasubject variability, but is of minimal importance when considering more stable parameters such as sperm DNA quality. A previous longitudinal study (Sergerie *et al.*, 2005) has reported that sperm DNA fragmentation measured by TUNEL analysis exhibits good within-individual stability over time, allowing for accurate assessment of baseline DNA damage from just a single entry sample.

The observation that antioxidant therapy was able to significantly reduce seminal ROS suggests that participants received a biologically adequate dose of antioxidant to positively influence the sperm DNA environment. Aside from a reduction in direct oxidative damage to the spermatozoa, the results of this study

suggest two other important mechanisms by which the Menevit® antioxidant may improve sperm DNA integrity. Firstly, a very significant reduction in sperm apoptosis was observed while on antioxidant therapy. Oxidative stress is known to initiate apoptosis (Moustafa *et al.*, 2004), which in turn will ultimately lead to endonuclease fragmentation of the sperm DNA. It is likely that by reducing free radical attack on good-quality spermatozoa, less of these spermatozoa will be forced down the path of abortive apoptotic DNA fragmentation. Secondly, the Menevit® antioxidant was shown to produce a small but significant improvement in sperm DNA protamination, making the sperm DNA more impregnable to ROS attack. As far as is known, the ADAM study is the first of its kind to show that sperm protamination and apoptosis can be positively influenced by the use of an oral antioxidant/ mineral supplement.

A previous study using a combinational antioxidant supplement (vitamin C 400 mg, vitamin E 400 mg, β-carotene 18 mg, zinc 500 µmol, selenium 1 µmol) has reported that antioxidants may adversely effect sperm chromatin condensation, possibly mediated by vitamin C interfering with inter-chain disulphide linkages in protamines (Ménézo *et al.*, 2007). These authors warn against the use of antioxidants in men with high degrees of sperm nuclear decondensation as protamine deficiency is linked with sperm premature chromosomal condensation and fertilization failure (Nasr-Esfahani, 2004). It is possible that the lower dose of vitamin C contained in the Menevit® supplement, together with the higher doses of zinc and selenium, may be responsible for the observed small improvement in sperm protamination compared with those observed in the Ménézo study. A previous placebo-controlled study (Tremellen *et al.*, 2007) has reported that the use of the Menevit® antioxidant slightly improves ICSI fertilization rates when compared with placebo or patients' own, historical, control IVF-ICSI cycles, making a significant negative effect of this antioxidant combination therapy on sperm nuclear condensation highly improbable. This is also supported by the good fertilization rates observed in the ADAM patients who did undergo IVF-ICSI treatment.

The active ingredients responsible for the observed improvement in sperm protamine content are most likely to be zinc and selenium as both have been associated with the process of sperm protamination. A link between zinc deficiency in men and abnormal sperm DNA condensation was first noted by Kvist *et al.* (1987). Similarly, rodents fed a zinc-deficient diet have been reported to have abnormal sperm chromatin packaging and a resulting increase in sperm DNA damage (Evenson *et al.*, 1993). Zinc enhances sperm DNA integrity by augmenting protamine 2 binding to the sperm DNA, thereby making the spermatozoa less susceptible to oxidative attack (Brewer *et al.*, 2002). Selenium is also an important cofactor in sperm DNA protamine condensation, with a 35 kDa seleno-protein being highly expressed late in spermatogenesis, and is felt to play an important role in disulphide cross-linking of protamines (Pfeifer *et al.*, 2001). As a deficiency in zinc and selenium is not uncommon within infertile men (Ebisch *et al.*, 2007), it is speculated that supplementation with zinc and selenium may have increased sperm protamine related DNA condensation, thereby making spermatozoa less susceptible to oxidative DNA damage.

The Menevit® antioxidant treatment had no significant effect on sperm count, motility or morphology. Previous small non-controlled studies have reported that lycopene (Gupta and Kumar, 2002), vitamin C and E (reviewed in Agarwal, 2004), and zinc and folate (Wong *et al.*, 2002) can improve sperm parameters such as count, motility and morphology. Four previous double-blind randomized controlled trials have examined the effect of antioxidant preparations (vitamin C, vitamin E, selenium combinations) on sperm parameters. Two (Rolf *et al.*, 1999; Greco *et al.*, 2005) found no significant effect of antioxidants on sperm count, motility or morphology, which is consistent with observations. Conversely, the remaining two randomized controlled trials found a small but significant improvement in sperm motility but no improvement in sperm count or morphology (Suleiman *et al.*, 1996; Keskes-Ammar *et al.*, 2003).

Several researchers have identified a significant correlation between sperm count, motility and morphology and sperm DNA integrity (Tremellen, 2008). Therefore it is uncertain why this study did not observe an improvement in these routine sperm parameters with antioxidant treatment, when sperm DNA integrity did improve. There are several possible explanations for this. Firstly, it is possible that sperm DNA is more resistant to ROS attack than the sperm membrane/mitochondria (determinants of motility). If oxidative attack is reduced by antioxidant therapy, yet not totally resolved, the concentrations of available ROS may no longer be sufficient to damage the DNA but are still capable of decreasing motility. Secondly, while previous studies have linked poor routine sperm parameters (count, motility and morphology) with high concentrations of ROS production, an association does not prove causation. Without a cause and effect link, antioxidant therapy cannot be expected to normalize all sperm parameters. For example, it has been well documented that excessive residual cytoplasm (abnormal morphology) is linked with an increase in the generation of ROS within spermatozoa because of increased cytoplasmic glucose-6-phosphate dehydrogenase activity (Said *et al.*, 2005). Here, abnormal morphology causes oxidative stress. However, while the use of antioxidants will reduce ROS within the spermatozoa, possibly reducing DNA damage, they cannot be expected to normalize sperm morphology. While the above statements are speculative, what is clear is that the majority of good-quality in-vivo studies do not show antioxidant therapy to have any material effect on routine sperm parameters (Tremellen, 2008), an observation which is consistent with these results.

Antioxidant treatment for 3 months had no significant effect on serum hormone concentrations, suggesting no change in the function of either the testicular Leydig cells (LH, testosterone) or Sertoli cells (FSH, AMH). Some investigators have speculated that oxidative damage to the Leydig cell's LH receptor is responsible for the gradual decline in serum testosterone that is observed with increasing male age (Hardy and Schlegel, 2004). If this is the case, some increase in serum testosterone or a drop in LH concentration would be expected, yet no such changes were observed. This lack of effect of the Menevit® antioxidant on reproductive hormones is consistent with the few examples within the existing literature. Comhaire *et al.* (2005) reported no change in serum LH, FSH or testosterone following 3 months of therapy with a potent carotenoid antioxidant (Astaxanthin®), despite recording a very significant fall in seminal ROS production. Similarly, a double-blinded randomized, placebo-controlled trial using a combination of zinc and folate reported no significant changes in serum testosterone or FSH (Wong *et al.*, 2002).

In conclusion, the results of the ADAM study suggest that previously reported improvements in pregnancy outcome during

IVF-ICSI treatment using the Menevit® antioxidant (Tremellen *et al.*, 2007) was most likely mediated by significant improvements in sperm DNA integrity. The current data suggest that the observed reduction in sperm DNA fragmentation is the net effect of a reduction in ROS attack, a reduction in DNA susceptibility to ROS damage because of improved sperm DNA protamine packaging, and a decline in ROS initiated apoptosis. As far as is known, the ADAM study is the first study to describe the ability of an oral antioxidant /mineral supplement to enhance sperm DNA protamination while reducing sperm apoptosis.

This study adds to the growing body of evidence supporting the use of antioxidant combinational therapy to improve sperm DNA integrity (Comhaire *et al.*, 2000; Greco *et al.*, 2005; Ménézo *et al.*, 2007), especially for those men undergoing IVF-ICSI treatment. Bypassing the quality control of natural fertilization by the use of ICSI enables fertilization to occur even in the presence of severely damaged sperm DNA, placing patients at significantly increased risk of miscarriage (Virro *et al.*, 2004; Borini *et al.*, 2006; Benchaib *et al.*, 2007). The clinical miscarriage rate of 18.8% (3/16) observed in this study's cohort was similar to that reported for fertile couples with no underlying sperm DNA quality issues (Hassold and Chiu, 1985). This observation suggests that treatment of men with high degrees of oxidative DNA damage with antioxidants before their partner commences IVF-ICSI therapy may be capable of improving pregnancy outcomes. Larger studies will be required to conclusively determine if preconception antioxidant supplements can reduce miscarriage rates.

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References

Agarwal A 2004 Role of antioxidants in treatment of male infertility: an overview of the literature. *Reproductive BioMedicine Online* **8**, 616–627.

Aitken RJ, De Iuliis GN 2007 Origins and consequences of DNA damage in male germ cells. *Reproductive BioMedicine Online* **14**, 727.

Aitken RJ, Gordon E, Harkiss D *et al.* 1998 Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. *Biology of Reproduction* **59**, 1037–1046.

Aoki VW, Moskovtsev SI, Willis J *et al.* 2005 DNA integrity is compromised in protamine-deficient human sperm. *Journal of Andrology* **26**, 741–748.

Baker HW, Kovacs GT 1985. Spontaneous improvement in semen quality: regression towards the mean. *International Journal of Andrology* **8**, 421–426.

Bakos HW, Thompson JG, Feil D, Lane M 2008 Sperm DNA damage is associated with assisted reproductive technology pregnancy. *International Journal of Andrology* **31**, 518–526.

Benchaib M, Lornage J, Mazoyer C *et al.* 2007 Sperm DNA fragmentation as a prognostic indicator of assisted reproductive technology outcome. *Fertility and Sterility* **87**, 93–100.

Benchaib M, Braun V, Lornage J *et al.* 2003 Sperm DNA fragmentation decreases the pregnancy rate in an assisted reproductive technique. *Human Reproduction* **18**, 1023–1028.

Bengum M, Humaidan P, Spano M *et al.* 2004 The predictive value of SCSA parameters for the outcome of intrauterine insemination, IVF and ICSI. *Human Reproduction* **19**, 1401–1408.

Borini A, Tarozzi N, Bizzaro D *et al.* 2006 Sperm DNA fragmentation: paternal effect on early post-implantation embryo development in ART. *Human Reproduction* **21**, 2876–2881.

Brewer L, Corzett M, Balhorn R 2002 Condensation of DNA by spermatid basic nuclear proteins. *Journal of Biological Chemistry* **41**, 38895–38900.

Carrell DT, Liu L 2001 Altered protamine 2 expression is uncommon in donors of known fertility, but common among men with poor fertilizing capacity, and may reflect other abnormalities of spermatogenesis. *Journal of Andrology* **22**, 604–610.

Choi HS, Kim JW, Cha YN, Kim C 2006 A quantitative nitroblue tetrazolium assay for determining intracellular superoxide anion production in phagocytic cells. *Journal of Immunoassay and Immunochemistry* **27**, 31–44.

Comhaire FH, El Garem Y, Mahmoud A *et al.* 2005 Combined conventional/ antioxidant 'Astaxanthin' treatment for male infertility: a double blind, randomized trial. *Asian Journal of Andrology* **7**, 257–262.

Comhaire FH, Christophe AB, Zalata AA *et al.* 2000 The effects of combined conventional treatment, oral antioxidants and essential fatty acids on sperm biology in subfertile men. *Prostaglandins, Leukotrienes and Essential Fatty Acids* **63**, 159–165.

Conrad M, Moreno SG, Sinowatz F *et al.* 2005 The nuclear form of phospholipid hydrogenperoxide glutathione peroxidase is a protein thiol peroxidase contributing to sperm chromatin stability. *Molecular and Cellular Biology* **25**, 7637–7644.

Ebisch IM, Thomas CM, Peters WH *et al.* 2007 The importance of folate, zinc and antioxidants in the pathogenesis and prevention of subfertility. *Human Reproduction Update* **13**, 163–174.

Esfandiari N, Sharma RK, Saleh RA *et al.* 2003 Utility of the nitroblue tetrazolium reduction test for assessment of reactive oxygen species production by seminal leukocytes and spermatozoa. *Journal of Andrology* **24**, 862–870.

Evenson DP, Emerick RJ, Jost LK *et al.* 1993 Zinc-silicon interactions influencing sperm chromatin integrity and testicular cell development in the rat as measured by flow cytometry. *Journal of Animal Science* **71**, 955–962.

Fujii J, Iuchi Y, Matsuki S, Ishii T 2003 Co-operative function of antioxidant and redox systems against oxidative stress in male reproductive tissues. *Asian Journal of Andrology* **5**, 231- 242.

Gandini L, Lombardo F, Paoli D *et al.* 2000 Study of apoptotic DNA fragmentation in human spermatozoa. *Human Reproduction* **15**, 830–839

Gomez E, Buckingham DW, Brindle J *et al.* 1996 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. *Journal of Andrology* **17**, 276–287.

Greco E, Iacobelli M, Rienzi L *et al.* 2005 Reduction of the incidence of sperm DNA fragmentation by oral antioxidant treatment. *Journal of Andrology* **26**, 349–353.

Gupta NP, Kumar R 2002 Lycopene therapy in idiopathic male infertility- a preliminary report. *International Urology and Nephrology* **34**, 369–372.

Hardy MP, Schlegel P 2004 Testosterone production in the aging male: Where does the slowdown occur? *Endocrinology* **145**, 4439–4440.

Hassold T, Chiu D 1985 Maternal age-specific rates of numerical chromosome abnormalities with special reference to trisomy. *Human Genetics* **70**, 11–17.

Heber D, Lu QY 2002 Overview of mechanism of action of lycopene. *Experimental Biology and Medicine* **227**, 920–923.

Henkel R, Kierspel E, Stalf T *et al.* 2005 Effect of reactive oxygen species produced by spermatozoa and leukocytes on sperm functions in non-leukospermic patients. *Fertility and Sterility* **83**, 635–642.

Henkel R, Mass G, Hajimohammad M *et al.* 2003 Urogenital inflammation: changes of leucocytes and ROS. *Andrologia* **35**, 309–313.

Hodge G, Hodge S, Han P 2002 Allium sativum (garlic) suppresses leukocyte inflammatory cytokine production in vitro: potential therapeutic use in the treatment of inflammatory bowel disease. *Cytometry* **48**, 209–215.

Keskes-Ammar L, Feki-Chakroun N, Rebaj T *et al*. 2003 Sperm oxidative stress and the effect of an oral vitamin E and selenium supplement on semen quality in infertile men. *Archives of Andrology* **49**, 83–94.

Kvist U, Bjorndahl L. Kjellberg S 1987 Sperm nuclear zinc chromatin stability and male fertility. *Scanning Microscopy* **1**, 1241–1247.

Larson KL, DeJong CJ, Barnes AM *et al*. 2000 Sperm chromatin structure assay parameters as predictors of failed pregnancy following assisted reproductive techniques. *Human Reproduction* **15**, 1717–1722.

Lewis SE, Aitken RJ 2005 DNA damage to spermatozoa has impacts on fertilization and pregnancy. *Cell and Tissue Research* **322**, 33–41.

Loft S, Kold-Jensen T, Hjollund NH *et al*. 2003 Oxidative DNA damage in human sperm influences time to pregnancy. *Human Reproduction* **18**, 1265–1272.

Manicardi GC, Bianchi PG, Pantano S *et al*. 1995 Presence of endogenous nicks in DNA of ejaculated human spermatozoa and its relationship to chromomycin A3 accessibility. *Biology of Reproduction* **52**, 864–867.

McLachlan R, de Krester D 2001 Male infertility. The case for continued research. *Medical Journal of Australia* **174**, 116–117.

Ménézo YJ, Hazout A, Panteix G *et al*. 2007 Antioxidants to reduce sperm DNA fragmentation: an unexpected adverse effect. *Reproductive BioMedicine Online* **14**, 418–421.

Mercardo-Pichardo E, Vilar-Rojas C, Bernal-Torres A.1981 Reduction capacity on nitroblue tetrazolium (NBT) of the normal human spermatozoa. *Archives of Medical Research* **12**, 107–114.

Moller P, Viscovich M, Lykkesfeldt J *et al*. 2004 Vitamin C supplementation decreases oxidative DNA damage in mononuclear blood cells of smokers. *European Journal of Nutrition* **43**, 267–274.

Moustafa MH, Sharma RK, Thornton J *et al*. 2004 Relationship between ROS production, apoptosis and DNA denaturation in spermatozoa from patients examined for infertility. *Human Reproduction* **19**, 129–138.

Nasr-Esfahani MH, Razavi S, Mozdarani H *et al*. 2004 Relationship between protamine deficiency with fertilization rate and incidence of sperm premature chromosomal condensation post-ICSI. *Andrologia* **36**, 95–100.

Ozmen B, Koutlaki N, Youssry M *et al*. 2007 DNA damage of human spermatozoa in assisted reproduction: origins, diagnosis, impacts and safety. *Reproductive BioMedicine Online* **14**, 384–395.

Pfeifer H, Conrad M, Roethlein D *et al*. 2001 Identification of a specific sperm nuclei selenoenzyme necessary for protamine thiol cross-linking during sperm maturation. *FASEB Journal* **15**, 1236–1238.

Rajpert-De Meyts E, Jorgensen N, Graem N *et al*. 1999 Expression of AMH during normal and pathological gonadal development: association with differentiation of sertoli and granulosa cells. *Journal of Clinical Endocrinology and Metabolism* **84**, 3836–3844.

Ramos L, van der Heijden GW, Derijck A *et al*. 2008 Incomplete nuclear transformation of human spermatozoa in oligo-astheno-teratospermia: characterization by indirect immunofluorescence of chromatin and thiol status. *Human Reproduction* **23**, 259–270.

Rolf C, Cooper TG, Yeung CH, Nieschlag E 1999 Antioxidant treatment of patients with asthenzoospermia or moderate oligoasthenozoospermia with high-dose vitamin C and vitamin E: a randomized, placebo-controlled, double-blind study. *Human Reproduction* **14**, 1028–1033.

Said TM, Agarwal A, Sharma RK *et al*. 2005 Impact of sperm morphology on DNA damage caused by oxidative stress induced by β-nicotinamide adenine dinucleotide phosphate. *Fertility and Sterility* **83**, 95–103.

Sakkas D, Seli E, Bizzaro D *et al*. 2003 Abnormal spermatozoa in the ejaculate: abortive apoptosis and faulty nuclear remodelling during spermatogenesis. *Reproductive BioMedicine Online* **7**, 428–432.

Saleh RA, Agarwal A, Kandirali E *et al*. 2002 Leukocytospermia is associated with increased reactive oxygen species production by human spermatozoa. *Fertility and Sterility* **78**, 1215–1224.

Saravanan G, Prakash J 2004 Effect of garlic (Allium sativum) on lipid peroxidation in experimental myocardial infarction in rats. *Journal of Ethnopharmacology* **94**, 155–158.

Seli E, Gardner DK, Schoolcraft WB *et al*. 2004 Extent of nuclear damage in ejaculated spermatozoa impacts on blastocyst development after in vitro fertilization. *Fertility and Sterility* **82**, 378–383.

Sergerie M, Laforest G, Boulanger K *et al*. 2005 Longitudinal study of sperm DNA fragmentation as measured by terminal uridine nick end-labelling assay. *Human Reproduction* **20**, 1921–1927.

Spano M, Bonde JP, Hjollund HI *et al*. 2000 Sperm chromatin damage impairs human fertility. *Fertility and Sterility* **73**, 43–50.

Suleiman SA, Ali ME, Zaki ZM *et al*. 1996 Lipid peroxidation and human sperm motility: protective role of vitamin E. *Journal of Andrology* **17**, 530–537.

Tarozzi N 2007 Clinical relevance of sperm DNA damage in assisted reproduction. *Reproductive BioMedicine Online* **14**, 746–759.

Tesarik J 2006 Sperm nuclear DNA damage: update on the mechanism, diagnosis and treatment. *Reproductive BioMedicine Online* **12**, 715–721.

Torregrosa N, Dominguez-Fandos D, Camejo MI *et al*. 2006 Protamine 2 precursors, protamine 1/ protamine 2 ratio, DNA integrity and other sperm parameters in infertile patients. *Human Reproduction* **21**, 2084–2089.

Tremellen K 2008 Oxidative stress and male infertility- a clinical perspective. *Human Reproduction Update* **14**, 243–258.

Tremellen K, Miari G, Froiland D, Thompson J 2007 A randomized control trial examining the effect of an antioxidant (Menevit) on pregnancy outcome during IVF-ICSI treatment. *Australian and New Zealand Journal of Obstetrics and Gynaecology* **47**, 216–221.

Tunc O, Thompson J, Tremellen K 2008 Development of the NBT assay as a marker of sperm oxidative stress. *International Journal of Andrology*. E-pub ahead of print, 2 December 2008.

Twigg J, Fulton N, Gomez E *et al*. 1998 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. *Human Reproduction* **13**, 1429–1436.

Valko M, Izakovic M, Mazur M *et al*. 2004 Role of oxygen radicals in DNA damage and cancer incidence. *Molecular and Cellular Biochemistry* **266**, 37–56.

Virro MR, Larson-Cook KL, Evenson DP 2004 Sperm chromatin structure assay (SCSA) parameters are related to fertilization, blastocyst development, and ongoing pregnancy in in vitro fertilization and intracytoplasmic sperm injection cycles. *Fertility and Sterility* **81**, 1289–1295.

Wong WY, Merkus HM, Thomas CM *et al*. 2002 Effects of folic acid and zinc sulfate on male factor subfertility: a double-blind, randomized, placebo-controlled trial. *Fertility and Sterility* **77**, 491–498.

World Health Organization 1999 *Laboratory Manual for the Examination of Human Semen and Sperm Cervical Mucus Interaction*, 4th edn. Cambridge University Press, Cambridge, UK.

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