The effect of ascorbate and α-tocopherol supplementation in vitro on DNA integrity and hydrogen peroxide-induced DNA damage in human spermatozoa

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The aim of this study was to determine the effects of supplementation with ascorbate and α-tocopherol, both singly and in combination, during sperm preparation on subsequent sperm DNA integrity, induced DNA damage and reactive oxygen species (ROS) generation. Semen samples with normozoospermic and asthenozoospermic profiles (n = 15 for each control and antioxidant group) were prepared by Percoll density centrifugation (95.0–47.5%) where the medium had been supplemented with these antioxidants to a number of different concentrations, all within physiological levels. Controls were included which had no ascorbate or α-tocopherol added. DNA damage was induced using hydrogen peroxide (H₂O₂) and DNA integrity was determined using a modified alkaline single cell gel electrophoresis (Comet) assay, while ROS generation was measured using chemiluminescence. Addition of ascorbate to sperm preparation medium did not affect baseline DNA integrity but did provide sperm with complete protection against H₂O₂-induced DNA damage. Generation of H₂O₂-induced ROS was also significantly reduced after treatment with ascorbate, although baseline levels were unaffected by this antioxidant. Supplementation of sperm preparation medium with α-tocopherol did not influence baseline DNA integrity but provided sperm with dose-dependent protection against H₂O₂-induced DNA damage. Generation of H₂O₂-induced ROS was significantly reduced after treatment with α-tocopherol, although baseline ROS levels were unaffected by this antioxidant. Addition of both ascorbate and α-tocopherol in combination to sperm preparation medium actually induced DNA damage and intensified the damage induced by H₂O₂, however, H₂O₂-induced ROS production was significantly reduced in a dose-dependent manner by supplementation with both vitamins.

Introduction

One in six couples will experience fertility problems at some stage in their reproductive lives, with the single most common cause of infertility cases presenting at out-patient clinics being spermatozoal dysfunction. It has been estimated that up to 40% of infertility cases are caused exclusively by a male factor (Fleming et al., 1995). In vitro fertilization with or without intra-cytoplasmic sperm injection (ICSI) is the most successful treatment for male factor infertility.

The introduction of ICSI, where an immobilized sperm is injected directly into the cytoplasm of the oocyte, has revolutionized the treatment of male infertility. ICSI bypasses all natural selection methods for determining which sperm will penetrate and ultimately fertilize an oocyte. Therefore, techniques such as computer-assisted semen analysis (CASA), which are used to quantitate sperm motion parameters, such as velocity and characteristics of track direction (Donnelly et al., 1998), as well as sperm morphology, have become somewhat redundant as the importance of these parameters has diminished. Hence, new techniques to assess sperm quality are urgently needed. One factor which still remains of the utmost importance for ICSI is sperm DNA integrity. Good quality sperm DNA is essential for the accurate transmission of genetic material to offspring. The DNA status of individual sperm can be determined using a modified alkaline single cell gel electrophoresis (Comet) assay (Hughes et al., 1996, 1997, 1998). This involves embedding sperm in agarose, lysing the membranes and breaking down the protein matrices. Broken strands of DNA are drawn out by electrophoresis to form a comet ‘tail’ leaving a ‘head’ of intact DNA. Intact and damaged DNA is quantified using epifluorescence microscopy and image analysis.

Sperm DNA is particularly vulnerable to damage induced by reactive oxygen species (ROS) (Steinken, 1989) due to the cell’s high content of polyunsaturated fatty acids and the absence of any repair mechanisms (Aitken and Clarkson, 1987; van Loon et al., 1991). The most obvious effect of free radical damage on sperm viability is membrane damage, which can occur through the accumulation of lipid peroxides (Aust, 1988; Hall and Brugher, 1988; Gutteridge, 1988) and also due to the loss of the diffusion barrier to membrane-impassable markers and to cell lysis (Imlay and Linn, 1988). Some protection from such oxidative insult is provided by seminal plasma. This maintains sperm cells in an environment which contains an abundance of antioxidant enzymes such as superoxide dismutase (SOD) and catalase, which removes key ROS such as O₂⁻ and hydrogen peroxide (H₂O₂) and scavengers such as albumin and taurine (Halliwell and Gutteridge, 1989), as well as crucial chain-breaking antioxidants such as urates, ascorbate and thiols.

During routine sperm preparation for use in assisted conception, sperm are separated from their seminal plasma in order to concentrate the subpopulation with the best morphology and motility. However, in doing so they are stripped of their antioxidant protection and left more vulnerable to oxidative attack. The addition of antioxidants such as ascorbate or α-tocopherol to sperm preparation medium to restore antioxidant protection has previously been shown to improve DNA integrity in human sperm and to afford protection against induced damage using X-ray radiation (Hughes et al., 1998), even when no free radical activity could be detected. The aim of this study was to modify the method used in the previous investigation using different decondensation agents to determine if baseline DNA integrity could be improved. It was also designed to determine if the addition of ascorbate and α-tocopherol, both singly and in combination, would improve

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DNA integrity and protect sperm DNA from the harmful effects induced by another DNA damaging agent, H₂O₂, using the modified Comet assay. Finally, to enhance the sensitivity of the Comet assay for sperm, our previous protocol was modified and lysis was performed before cells were exposed to the DNA damaging agent.

Materials and methods

Collection of semen samples

Semen samples were obtained from a total of 45 men whose semen profiles were either normozoospermic (n = 12, concentration ≥ 20x10⁶/ml, progressive motility ≥ 50%) or asthenozoospermic (n = 33, concentration ≥ 20x10⁶/ml, progressive motility ≤ 30%); World Health Organization, 1992) for a minimum of 3 days and a maximum of 7 days sexual abstinence. All subjects were the partners of women who had failed to conceive after 2 years of unprotected intercourse. Informed consent for participation was obtained and the project was approved by the Queen’s University of Belfast Research and Ethics Committee. Within 1 h of production, a routine semen analysis was performed using light microscopy to determine concentration, motility and morphology.

Preparation of samples

Samples were prepared using a two-step discontinuous Percoll gradient (95.0–47.5%; Pharmacia Biotech AB, Uppsala, Sweden). Each aliquot of liquefied semen was layered on top of the gradient and centrifuged at 450 g for 12 min. The resulting sperm pellet was centrifuged by centrifugation at 200 g for 6 min. The final sperm preparation was suspended in a suitable volume of Biggers, Whitten and Whittingham medium (BWW; Biggers et al., 1971) supplemented with 600 mg of albumin (Alpha Therapeutic UK Ltd, Norfolk, UK) and the required antioxidant as appropriate. Post-Percoll concentrations were adjusted to 1x10⁷/50 µl using BWW medium and motilities were determined for all samples using 20 µm depth Microcell counting chambers (Conception Technologies Inc., La Jolla, CA).

Antioxidant treatment

Sperm from each of the 45 patients was divided into three aliquots. One aliquot acted as a control and was prepared by Percoll density centrifugation as previously described. The remaining two aliquots were prepared by Percoll in the presence of either ascorbate or α-tocopherol (in the form of Trolox) at one of two different concentrations. Trolox is a water-soluble analogue of α-tocopherol, the hydrophobic side chain being replaced by a hydrophilic –COOH group. It is a powerful antioxidant (Barclay et al., 1984) which has been shown to protect mammalian cells from oxidative damage both in vivo (Mickle et al., 1989) and in vitro (Forrest et al., 1994). Ascorbate and/or α-tocopherol was added to all of the preparation medium used subsequently for that aliquot (i.e. both layers of Percoll and BWW medium) as follows:

(i) ascorbate (L-ascorbic acid; Sigma Aldrich Co. Ltd, Poole, UK) at concentrations of 300 and 600 µM; (ii) α-tocopherol (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid, Trolox; Sigma Aldrich) at 40 and 60 µM; (iii) ascorbate + α-tocopherol (at 600 + 60 and 300 + 40 µM). These concentrations of antioxidants span the range found in blood and seminal plasma from fertile men (Lewis et al., 1997). Antioxidants were present throughout the preparation procedure. Fifteen patients (four normozoospermic and 11 asthenozoospermic) were treated with ascorbate, 15 were treated with α-tocopherol and 15 with a combination of ascorbate + α-tocopherol.

Determination of DNA integrity using a modified alkaline single cell gel electrophoresis (Comet) assay

The following procedure (adapted from Hughes et al., 1996, 1997, 1998) was carried out under yellow light to prevent further induced damage to DNA.

Embedding of sperm in agarose gel

Fully frosted microscope slides (Richardssons Supply Co. Ltd, London, UK) were covered with 100 µl of 0.5% normal melting point agarose in Ca²⁺-, and Mg²⁺-free phosphate-buffered saline (PBS; Sigma Aldrich) at <45°C and immediately covered with a large coverslip. When the agarose had solidified, the coverslips were removed and 1x10⁷ sperm in 10 µl BWW were mixed with 75 µl of 0.5% low melting point agarose at 37°C. This cell suspension was rapidly pipetted on top of the first agarose layer, covered with a coverslip and allowed to solidify at room temperature. Two slides were prepared for each control and each test antioxidant to ensure that DNA damage could be induced at a later stage while still retaining an undamaged replicate. A total of six slides from each patient was therefore obtained: control; sperm to be treated with H₂O₂ at a later stage in the protocol; sperm treated with a low concentration of antioxidant; sperm treated with a low concentration of antioxidant plus H₂O₂ at a later stage; sperm treated with a high concentration of antioxidant; sperm treated with a high concentration of antioxidant plus H₂O₂ at a later stage.

Lysing of cells and decondensation of DNA

The coverslips were removed and the slides were immersed in a coplin jar containing freshly prepared cold lysing solution [2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris, pH 10, with 1% Triton X-100 (Sigma) added just before use] for 1 h at 4°C. Slides were then incubated for 30 min at 4°C with 10 mM dithiothreitol (DTT; Sigma) followed by 90 min incubation at 20°C with 4 mM lithium diiodosalicylate (LIS; Sigma) (Robbins et al., 1993). Therefore, the total time taken for lysis and decondensation of DNA is 3 h, which is a modification of our previous methodology where cells were incubated overnight (~12 h) at 37°C with 0.1 mg/ml proteinase K added to the lysis solution (Hughes et al., 1996, 1997, 1998). Test slides had the required antioxidant present throughout these lysis and decondensation steps.

Induction of DNA damage using H₂O₂

DNA damage was then induced using H₂O₂ in one control and test slide for each antioxidant. This is another modification of our previous protocol, where damage is now induced after lysis and decondensation of DNA. Slides were immersed in a coplin jar containing 200 µM H₂O₂ (Sigma) in PBS and incubated at 4°C for 1 h. Corresponding control slides were placed in a coplin jar containing only PBS and incubated under the same conditions.

Unwinding of DNA

The slides were removed from the lysis solution + DTT + LIS and carefully drained of any remaining liquid. A horizontal gel electrophoresis tank was filled with fresh alkaline electrophoresis solution at 12–15°C containing 300 mM NaOH and 1 mM EDTA (Sigma). The slides were placed into this tank side by side with the agarose end facing the anode and with the electrophoresis buffer at a level of ~0.25 cm above the slide surface. The slides were left in this high pH buffer for 20 min to allow DNA in the cells to unwind.

Separation of DNA fragments by electrophoresis

Electrophoresis was conducted for 10 min at 25 V (0.714 V/cm) adjusted to 300 mV by raising or lowering the buffer level in the tank. After electrophoresis the slides were drained, placed on a tray and flooded with three changes of neutralization buffer (0.4 M Tris, pH 7.5; Sigma) for 5 min each. This removes any remaining alkali and detergents which would interfere with ethidium bromide staining. The slides were then drained before being stained with 50 µl of 20 µg/ml ethidium bromide (Sigma) and covered with a coverslip.

Image analysis

The slides were viewed using a Nikon E600 epifluorescence microscope which was equipped with a 515–560 nm excitation filter, a 100 W mercury lamp and a 590 nm barrier filter. Fifty images were captured and analysed by an image analysis system using the program Komet 3.1.

Modifications to the ‘Comet’ assay protocol

Modification of our previous method to decondense DNA using DTT and LIS rather than proteinase K and a reduced lysis and decondensation time of 3 rather than 12 h resulted in a significant improvement in the values observed for baseline sperm DNA integrity. Values of ~70–75% undamaged head DNA were recorded in earlier studies where proteinase K was employed (Hughes et al., 1996, 1997, 1998), compared with a mean value of 90% in the present study.

In the current study we lysed the cells and decondensed the DNA before inducing further damage with H₂O₂, whereas the aforementioned investigations damaged the sperm before the lysis and decondensation steps. Our modification made the DNA more sensitive to damage following H₂O₂ treatment (see Results).

Measurement of ROS production

ROS were measured with a Lumat LB9507 luminometer (Berthold, Wildbad, Germany) using a modification of the method of Krausz et al. (1992).

Preparation of samples

Sperm samples (n = 15 for each antioxidant) were prepared by Percoll density centrifugation with and without antioxidant supplementation. Sperm from the 95% Percoll layer were removed and the concentration adjusted to 1x10⁷/ml. ROS activity was measured in control samples and following 2 and 4 h incubation with 600 µM ascorbate, 60 µM α-tocopherol and with both these antioxidants in combination. To determine the effects of these antioxidants on H₂O₂-induced ROS generation, control and antioxidant-treated samples were exposed to 200 µM H₂O₂ for 1 h at 4°C.

Measurement of ROS production

Background activity in a 200 µl aliquot of prepared sperm at a concentration of 1x10⁷/ml was measured over a 5 min period. Sperm were then incubated with 25 µM 5-amino-2,3-dehydro-1,4 phthalalizinedione (luminol; Sigma) sup-
Supplementation with 12.4 U horseradish peroxidase (Type VI, 310 mg/ml; Sigma) for a further 10 min to capture the basal luminol-dependent signal. The cells were stimulated with 50 µM N-formyl-methionyl-leucyl-phenylalanine (FMLP) in order to stimulate free radical production by leukocytes. The response was recorded for 20 min to measure the extent of the peak chemiluminescence response which was likely to be attributable to seminal leukocytes and to allow the system to settle back to baseline values. The sperm were then stimulated with 100 nm 12-myristate 13-acetate phorbol ester (PMA) and recorded for an additional 15 min to determine the total free radical production by both seminal leukocytes and sperm in the semen sample (Krausz et al., 1992). The residual capacity of the sperm population for free radical production was determined by subtracting the reading obtained after FMLP stimulation from that recorded after PMA stimulation, adjusting the value to allow for background interference. Values of >100 relative light units were considered to be indicative of ROS generation by sperm.

**Statistical analysis**

Results were analysed using Statistica 5.0 (Statsoft Europe, Hamburg, Germany). In view of the non-Gaussian distribution of data, the non-parametric Wilcoxon matched pairs test was employed to determine the effects of antioxidant supplementation on sperm DNA integrity. Results were considered to be statistically significant if a P value of <0.05 was recorded.

**Results**

**Details of samples used**

Of the 45 samples used, the mean percent progressive motility of the normozoospermic samples (n = 12) was 54.5%, compared with 28.6% for asthenozoospermic samples (n = 33) (P ≤ 0.05; data not shown). There was no significant difference in baseline DNA integrity between the normo- and asthenozoospermic groups (P > 0.05; data not shown).

**Effect of ascorbate supplementation in vitro (300 and 600 µM) on DNA integrity and H2O2-induced DNA damage in human sperm**

Supplementation of sperm preparation medium with ascorbate at both 300 and 600 µM had no significant effect on baseline DNA integrity (P > 0.05). Percentage intact head DNA was significantly reduced by 50% in sperm exposed to 200 µM H2O2 (P ≤ 0.005) and ascorbate supplementation at both 300 and 600 µM provided cells with complete protection against this H2O2-induced DNA damage (P ≤ 0.005; Fig. 1).

**Effect of α-tocopherol supplementation in vitro (40 and 60 µM) on DNA integrity and H2O2-induced DNA damage in human sperm**

Supplementation with α-tocopherol had no significant effect on sperm baseline DNA integrity (P > 0.05). However, it did provide significant dose-dependent protection against H2O2-induced DNA damage (P ≤ 0.005; Fig. 2).

**Effect of supplementation with a combination of ascorbate + α-tocopherol in vitro (300 + 40 and 600 + 60 µM) on DNA integrity and H2O2-induced DNA damage in human sperm**

Supplementation of preparation medium with ascorbate + α-tocopherol at both concentrations caused significant DNA damage in controls (P ≤ 0.01; Fig. 3) and exacerbated the DNA damage which was induced by H2O2 (P ≤ 0.005; Fig. 3).

**Effect of ascorbate supplementation in vitro (300 and 600 µM) on baseline and H2O2-induced free radical production by sperm**

Ascorbate at 300 and 600 µM did not have any effect on basal ROS generation but significantly reduced H2O2-induced free radical production by sperm in a dose-dependent manner (P ≤ 0.005; Fig. 4).

**Effect of α-tocopherol supplementation in vitro (40 and 60 µM) on baseline and H2O2-induced free radical production by sperm**

Similarly, α-tocopherol at concentrations of 40 and 60 µM inhibited H2O2-induced ROS production in a dose-dependent manner (P ≤ 0.005; Fig. 5), but had no effect on basal levels of ROS.

**Effect of supplementation with a combination of ascorbate + α-tocopherol in vitro (300 + 40 and 600 + 60 µM) on baseline and H2O2-induced free radical production by sperm**

Addition of ascorbate + α-tocopherol in combination in vitro also significantly reduced H2O2-induced ROS production in a dose-dependent manner (P ≤ 0.005; Fig. 6).
Fig. 3. The effect of supplementation in vitro with 300 \( \mu M \) ascorbate + 40 \( \mu M \) \( \alpha \)-tocopherol and 600 \( \mu M \) ascorbate + 60 \( \mu M \) \( \alpha \)-tocopherol on human sperm DNA integrity. (A) Control. (B) Sperm treated with 300 \( \mu M \) ascorbate + 40 \( \mu M \) \( \alpha \)-tocopherol. (C) Sperm treated with 600 \( \mu M \) ascorbate + 60 \( \mu M \) \( \alpha \)-tocopherol. (D) Sperm treated with 200 \( \mu M \) \( H_2O_2 \). (E) Sperm treated with 300 \( \mu M \) ascorbate + 40 \( \mu M \) \( \alpha \)-tocopherol + 200 \( \mu M \) \( H_2O_2 \). (F) Sperm treated with 600 \( \mu M \) ascorbate + 60 \( \mu M \) \( \alpha \)-tocopherol + 200 \( \mu M \) \( H_2O_2 \). Values are medians (□) with interquartile ranges (□ 25–75%) and minimum–maximum values (I). *Significantly greater than control value \(( P < 0.005, \text{Wilcoxon matched pairs test})\). **Significantly less than control value \(( P < 0.005, \text{Wilcoxon matched pairs test})\). † Significantly less than value for sample treated with \( H_2O_2 \) only \((-0.005, \text{Wilcoxon matched pairs test})\).

Discussion

Good quality sperm DNA is essential for the accurate transmission of genetic material. This has become even more relevant since the introduction of assisted conception techniques such as ICSI, where there are no longer natural barriers involved to exclude defective DNA from entering the oocyte. Poor quality DNA may not necessarily prevent fertilization from occurring and it has been shown that genetically damaged human sperm are still able to form normal pronuclei in oocytes after ICSI (Twigg et al., 1998). Therefore, the first indication of an existing DNA problem may be the occurrence of congenital abnormalities in offspring.

The DNA status of individual sperm is determined using our modified Comet assay and this study has used a further variation of a previous method. We found that lysis and decondensation of DNA over a 3 h period using a combination of DTT and LIS (Robbins et al., 1993), rather than overnight incubation with proteinase K, resulted in a greater baseline damage that was induced by overnight incubation with

Fig. 4. The effect of supplementation in vitro with 300 and 600 \( \mu M \) ascorbate on ROS production by human sperm. (A) Control. (B) Sperm treated with 600 \( \mu M \) ascorbate. (C) Sperm treated with 200 \( \mu M \) \( H_2O_2 \). (D) Sperm treated with 600 \( \mu M \) ascorbate + 200 \( \mu M \) \( H_2O_2 \). (E) Sperm treated with 600 \( \mu M \) ascorbate + 60 \( \mu M \) \( \alpha \)-tocopherol. Values are medians (□) with interquartile ranges (□ 25–75%) and minimum–maximum values (I). *Significantly greater than control value \(( P < 0.005, \text{Wilcoxon matched pairs test})\). † Significantly less than control value \(( P < 0.005, \text{Wilcoxon matched pairs test})\). ‡ Significantly less than value for sample treated with \( H_2O_2 \) only \((-0.005, \text{Wilcoxon matched pairs test})\).

Fig. 5. The effect of supplementation in vitro with 30 and 60 \( \mu M \) \( \alpha \)-tocopherol on ROS production by human sperm. (A) Control. (B) Sperm treated with 60 \( \mu M \) \( \alpha \)-tocopherol. (C) Sperm treated with 200 \( \mu M \) \( H_2O_2 \). (D) Sperm treated with 40 \( \mu M \) \( \alpha \)-tocopherol + 200 \( \mu M \) \( H_2O_2 \). (E) Sperm treated with 60 \( \mu M \) \( \alpha \)-tocopherol + 200 \( \mu M \) \( H_2O_2 \). Values are medians (□) with interquartile ranges (□ 25–75%) and minimum–maximum values (I). *Significantly greater than control value \(( P < 0.005, \text{Wilcoxon matched pairs test})\). † Significantly less than value for sample treated with \( H_2O_2 \) only \((-0.005, \text{Wilcoxon matched pairs test})\).

Fig. 6. The effect of supplementation in vitro with 300 \( \mu M \) ascorbate + 40 \( \mu M \) \( \alpha \)-tocopherol and 600 \( \mu M \) ascorbate + 60 \( \mu M \) \( \alpha \)-tocopherol on ROS production by human sperm. (A) Control. (B) Sperm treated with 600 \( \mu M \) ascorbate + 60 \( \mu M \) \( \alpha \)-tocopherol. (C) Sperm treated with 200 \( \mu M \) \( H_2O_2 \). (D) Sperm treated with 300 \( \mu M \) ascorbate + 40 \( \mu M \) \( \alpha \)-tocopherol + 200 \( \mu M \) \( H_2O_2 \). (E) Sperm treated with 600 \( \mu M \) ascorbate + 60 \( \mu M \) \( \alpha \)-tocopherol + 200 \( \mu M \) \( H_2O_2 \). Values are medians (□) with interquartile ranges (□ 25–75%) and minimum–maximum values (I). *Significantly greater than control value \(( P < 0.005, \text{Wilcoxon matched pairs test})\). † Significantly less than value for sample treated with \( H_2O_2 \) only \((-0.005, \text{Wilcoxon matched pairs test})\).
The effects of antioxidant supplementation, or damaging agents, on sperm DNA integrity in these previous studies were all related to controls subjected to the same protocol and therefore their conclusions remain valid. These earlier studies also involved induction of DNA damage in sperm before proceeding with membrane lysis and decondensation of DNA (Hughes et al., 1996, 1998). In the current investigation, a second modification of this protocol involved damaging DNA with H₂O₂ after performing the lysis and decondensation steps. We observed a decrease of ~50% in head DNA after exposure of sperm to 200 µM H₂O₂ using this amended method. This is a greater decrease than that observed previously by Hughes et al. (1996), where a reduction of ~40% in head DNA was observed. Therefore, we have increased the sensitivity of the Comet assay by decondensing and relaxing the tightly bound DNA within the sperm head nucleus before attempting to induce further DNA damage.

Using these modifications of the Comet assay we extended previous studies using X-ray irradiation (Hughes et al., 1998) to investigate the effect of supplementation of sperm preparation medium in vitro with ascorbate and α-tocopherol on baseline DNA integrity and induced DNA damage using H₂O₂. X-ray irradiation damages DNA by ionizing water to produce HO· radicals and by directly inducing single- and double-strand breaks in DNA. ROS such as HO·, O₂·– and H₂O₂ attack almost all cell components, including DNA, proteins and lipid membranes, and are capable of causing lethal damage to cells (Inoue et al., 1993).

Superoxide has the ability to act as both oxidant and reductant, but it is thought that its cytotoxic nature is most probably mediated by H₂O₂ and its ability to generate the intracellular HO· radical. Both O₂·– and HO· radicals have been implicated in a range of toxic effects. They are known to be mutagenic and cause chromosome deletions, dicentrics and sister chromatid exchanges (Larramendy et al., 1983; Chesis et al., 1984). They can also attack DNA at either the sugar or the base, giving rise to a number of products, such as methylarthonylurea, urea and 5-hydroxymethyluracil (Hutchinson, 1985; Imlay and Linn, 1988). Attack at the sugar ultimately results in fragmentation, base loss and a strand break with a terminal fragmented sugar residue.

Hydrogen peroxide is known to produce ring saturated thymines, hydroxymethyl uracil, thymine fragments and adenine ring-opened products by oxygen radical attack at DNA bases (Dempel and Linn, 1982; Breimer and Lindahl, 1985). However, sperm treated with H₂O₂ to the point where they become immobilized, and therefore demonstrably affected by oxidative stress, can still undergo a normal cycle of nuclear decondensation and pronucleus formation following ICSI (Twigg et al., 1998).

Exogenous ROS generation has been shown to cause a 4-fold increase in DNA fragmentation in human sperm after preparation by swim-up using the method of terminal deoxynucleotidyl transferase (TdT)-mediated DNA end-labelling (TUNEL) (Lopes et al., 1998a). Administration of antioxidants reduced this DNA fragmentation, with reduced glutathione and hypotaurine providing the most protection (Lopes et al., 1998a).

There has recently been much discussion as to the potential advantages of antioxidant therapy to improve male fertility (Ford and Whittingham, 1998; Lenzi et al., 1998; Tarin et al., 1998). However, it is recognized that antioxidant therapy may be a double-edged sword with undesirable effects if a safety threshold dosage is surpassed (Tarin et al., 1998). However, there are many potential advantages to antioxidant supplementation both in vivo and in vitro and our current study outlines the beneficial effects of antioxidant supplementation on induced DNA damage.

Using the Comet assay, we have shown here that ascorbate (300 and 600 µM) or α-tocopherol supplementation (40 and 60 µM) over a 3 h period did not lead to any improvement in baseline sperm DNA integrity. These antioxidants both provided protection against H₂O₂-induced DNA damage, although the protective effect with α-tocopherol was not as marked as that observed for ascorbate. There was also a significant reduction in H₂O₂-induced free radical production by sperm following antioxidant treatment.

Ascorbate or α-tocopherol (added in isolation) have previously been found to improve both baseline sperm DNA integrity and to decrease the level of damage observed after X-ray irradiation (Hughes et al., 1998). The most probable reason for the lack of improvement in basal DNA integrity here is that our baseline values were considerably higher than those recorded in the aforementioned study due to our modification of the Comet assay protocol. Our observation that H₂O₂-induced DNA damage is decreasing by ascorbate and α-tocopherol is in agreement with the previous findings for X-ray irradiation (Hughes et al., 1998). Hence, even our short term (3 h) exposure to these antioxidants confers equal protection against subsequent DNA damage as the overnight exposure previously reported (Hughes et al., 1998).

These findings may have clinical implications. It has been reported that poor quality sperm, such as those used for ICSI, have a greater percentage of sperm with DNA fragmentation than normal fertile samples (Sun et al., 1997; Lopes et al., 1998b). Previous studies from our laboratory found that baseline levels of DNA damage were similar in fertile and infertile men, although DNA from the infertile group were considerably more susceptible to damaging agents such as X-ray irradiation and H₂O₂ (Hughes et al., 1996). Supplementation of preparation medium with ascorbate or α-tocopherol may reduce free radical production and decrease ROS-induced DNA damage in patients with poor quality sperm. This in turn may provide a greater chance of successful fertilization, as there is an inverse correlation between the percentage of sperm with DNA fragmentation and fertilization rates in vitro with both IVF (Sun et al., 1997) and ICSI (Lopes et al., 1998b). There is also the possibility that oral administration of ascorbate may facilitate a reduction in induced DNA damage, although this is an area which requires further investigation before any firm conclusions can be drawn.

Ascorbate is known to act as a scavenger of a wide range of ROS (Sies et al., 1992), which explains its ability to successfully counteract the effects of H₂O₂ both in terms of induced DNA damage and ROS production. It has previously been shown to be the major antioxidant in seminal plasma of fertile men, contributing up to 65% of the total chain-breaking antioxidant capacity (Lewis et al., 1995). In addition, the concentration of this antioxidant in seminal plasma is 10 times greater than the concentration found in blood plasma (Lewis et al., 1997). Ascorbate is actually secreted from seminal vesicles during ejaculation (Berg et al., 1941) and it has been suggested that dietary ascorbate can protect human sperm from endogenous oxidative DNA damage (Fraga et al., 1991). In agreement with our study, it has been reported that ascorbate has been shown to provide a small protective effect on DNA integrity against H₂O₂-induced damage at doses of <200 µM.
in human lymphocytes, although it exacerbates DNA damage at higher doses (1–5 mM; Anderson et al., 1994).

Previous studies have shown that α-tocopherol affords mammalian cells some protection from oxidative attack in both in vivo (Casini et al., 1985; Mickle et al., 1989) and in vitro (Wu et al., 1990) studies. Oral administration of this antioxidant (200 mg daily for 3 months) has been found to improve the in vitro fertilization rate of fertile normozoospermic males with low fertilization rates in previous IVF cycles after 1 month of treatment (Geva et al., 1996). This may be due to the antioxidant acting by a reduction in lipid peroxidation and without any effect on the quantitative ultramorphology of subcellular organelles (Geva et al., 1996). Oral administration of vitamin E has also been shown to lead to a significant improvement in the in vitro function of human sperm as assessed using the zona-binding test (Kessopoulou et al., 1995) and has been suggested as a treatment for ROS-associated male infertility. In terms of DNA integrity, α-tocopherol was found to have no protective effect against H₂O₂ or bleomycin (Anderson et al., 1994) when assessed using the Comet assay.

α-Tocopherol is present in small quantities in seminal plasma (0.08–0.9 µmol/l; Lewis et al., 1997), but this may be due to the regeneration of this antioxidant by ascorbate (Kagan, 1992). α-Tocopherol and ascorbate work synergistically to protect against lipid peroxidation, with ascorbate recycling α-tocopherol and allowing it to again function as a free radical chain-breaker (Buettner, 1993). Despite this, we found that addition of both ascorbate and α-tocopherol to sperm preparation medium caused DNA damage in controls and exacerbated the DNA damage which was induced by H₂O₂. This is in agreement with previous findings by Hughes et al. (1998), who observed the same effect for controls and X-ray irradiated samples. In addition, an in vivo pilot study (Hughes et al., unpublished data) found supplementation with ascorbate + α-tocopherol together for 1 month induced DNA damage in human sperm.

Similarly, other instances have been reported where the addition of flavonoids (which are known to have antioxidant-like properties; Anderson et al., 1994; Pietrangelo et al., 1995) to sperm in vitro caused DNA damage (Anderson et al., 1997). The flavonoids silimarin, myricetin, quercetin, kaempferol, rutin and kaempferol-3-rutinoside were investigated in combination with the food mutagens 3-amino-1-methyl-5H-pyrido[4,3-b]indole and 2-amino-3-methylimidazo-4,5-quinoline using the Comet assay. The flavonoids were found to modulate the effects of the food mutagens in human lymphocytes and sperm and also showed exacerbating effects at low doses and antigenotoxic effects at high doses (Anderson et al., 1997).

Other groups have also reported the same detrimental effect for human lymphoblastoid cells where treatment with ascorbate and α-tocopherol in combination did not give additional protection over single antioxidant supplements to DNA from X-ray and H₂O₂ treatment in vitro (Sweetman et al., 1997). It has been suggested that a tightly regulated optimal level may exist for these two vitamins in combination, outside of which there is no added benefit or protective effect against free radicals (Sweetman et al., 1997). However, in our current study, addition of both antioxidants in combination in vitro significantly reduced H₂O₂-induced ROS production, therefore the observed increase in DNA damage caused by H₂O₂ plus ascorbate and α-tocopherol was not likely to be caused by these vitamins acting as pro-oxidants.

This study has shown that when DNA is ≈80% intact, the addition of ascorbate or α-tocopherol to sperm preparation medium does not significantly improve baseline DNA integrity in human sperm. However, these antioxidants still provide such sperm with protection against subsequent H₂O₂-induced DNA damage and also cause a significant decrease in H₂O₂-induced ROS production. This may therefore be useful in preparation of good quality sperm for ICSI where high levels of ROS have previously been detected. This is certainly a rapidly expanding area with great potential for the treatment of male infertility that can be attributable to excess ROS production.

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References


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