Glutathione and hypotaurine *in vitro*: effects on human sperm motility, DNA integrity and production of reactive oxygen species

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Sperm DNA integrity is of paramount importance for the accurate conveyance of genetic material. DNA damage may be a major contributory factor in male infertility as DNA from sperm of infertile men has been found to be more susceptible to induced DNA damage *in vitro* than DNA from fertile men. Reactive oxygen species (ROS) are a significant source of DNA damage and human sperm are extremely sensitive to ROS attack due to their high content of polyunsaturated fatty acids and lack of capacity for DNA repair. Seminal plasma, which contains a wealth of antioxidants, provides sperm with crucial protection against oxidative insult. However, during preparation for use in assisted conception techniques, sperm are separated from seminal plasma and deprived of that essential protection. The aim of this study was to determine the effects of supplementation with glutathione and hypotaurine during sperm preparation on subsequent sperm motility, DNA integrity, induced DNA damage and ROS generation. Semen samples ($n = 45$) were divided into aliquots and prepared by Percoll density centrifugation (95.0–47.5%) using medium which had been supplemented with these antioxidants to a number of different concentrations all within physiological levels. Control aliquots were included which had no glutathione or hypotaurine added. Sperm motility was determined using computer-assisted semen analysis. DNA damage was induced using H_2O_2 and DNA integrity was determined using a modified alkaline single cell gel electrophoresis (Comet) assay, while ROS generation was measured using chemiluminescence. Addition of glutathione and hypotaurine, either singly or in combination, to sperm preparation medium had no significant effect on sperm progressive motility or baseline DNA integrity. Despite this, sperm were still afforded significant protection against H_2O_2-induced damage and ROS generation.

Introduction

Methods such as conventional and computer-assisted semen analysis (CASA) have been, and are still, widely used as a means of determining the quality of a semen sample. Sperm CASA parameters such as progressive motility and average path velocity have recently been shown to provide a useful indicator of the potential of sperm to achieve fertilization *in vitro* and pregnancy (Donnelly *et al.*, 1998).

The introduction of intracytoplasmic sperm injection (ICSI) where a single immobilized sperm is injected directly into the cytoplasm of the oocyte has revolutionized the treatment of severe male factor infertility. Sperm DNA integrity is of the utmost importance for ICSI. Good quality sperm DNA is essential for the accurate transmission of genetic material. Poor quality DNA may not necessarily prevent fertilization from occurring and the first indication of an existing DNA problem may be the occurrence of congenital abnormalities in offspring.

Sperm DNA is particularly vulnerable to damage induced by reactive oxygen species (ROS) (Steenken, 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). Some protection from such oxidative insult is provided by seminal plasma, which contains an abundance of antioxidant enzymes such as superoxide dismutase (SOD) and catalase, which removes key ROS such as $O_2^{·-}$ and H_2O_2. Seminal plasma also contains scavengers such as albumin and taurine (Halliwell and Gutteridge, 1989) as well as crucial chainbreaking antioxidants such as urates, ascorbate and thiol groups.

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 media to restore antioxidant protection has previously been shown to provide sperm with added protection against induced damage using H_2O_2 (Donnelly *et al.*, 1999a) and X-irradiation (Hughes *et al.*, 1998). This study was designed to extend this work to examine the effect of supplementation with other antioxidants, namely glutathione and hypotaurine, on sperm function.

Reduced glutathione (GSH) is present in many organs and tissues of the body and has many antioxidant properties (Meister, 1992). It is the substrate of glutathione peroxidase (GPX) and plays a prominent role in the body's antioxidant defences. Despite the fact that high levels (1000 µg/g tissue) of GSH are found in the testis of rats (Vina *et al.*, 1992) and the reproductive tract fluids and epididymal sperm of bulls (Agrawi and Vahna-Perttula, 1988), much lower levels are found in the ejaculate (171 µg/10^6 rat sperm). This may indicate that despite the fact that GSH is an important antioxidant within the reproductive tract (Den Boer *et al.*, 1989, 1990) it is not released in the ejaculate, where other antioxidants such as ascorbate are much more abundant (Lewis *et al.*, 1997).

Hypotaurine (2-aminoethane sulfinic acid) is a precursor of taurine (2-aminoethane sulfonic acid), which is the main end product of cysteine metabolism in mammals (Huxtable, 1992). Taurine and hypotaurine are present in most mammalian tissues (Huxtable, 1992). They have been identified in mammalian sperm and reproductive tract (van der Horst and Grooten, 1966; van der Horst and Brand, 1969; Kochakian, 1973) and seem to be essential for sperm capacitation (Meizel *et al.*, 1980), motility (Boatman *et al.*, 1990), fertilization and early

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embryo development (Leibfried and Bavister, 1981; Barnett and Bavister, 1992). In human sperm there is an apparent association between hypotaurine content and fertility (Holmes *et al.*, 1992).

Therefore, the aim of this study was to determine the effects of the addition of the antioxidants glutathione and hypotaurine, both singly and in combination, on subsequent sperm motility, DNA integrity, induced DNA damage and ROS generation.

Materials and methods

Collection of semen samples

Semen samples were obtained from a total of 45 men whose semen profiles were either normozoospermic ($n = 24$, concentration $\geqslant 20 \times 10^6$/ml, progressive motility $\geqslant 50\%$) or asthenozoospermic ($n = 21$, concentration $\geqslant 20 \times 10^6$/ml, progressive motility $< 50\%$) (World Health Organization, 1992) following a recommended 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 concentrated 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 albuten (Alpha Therapeutic UK, Norfolk, UK) and the required antioxidant as appropriate.

Antioxidant supplementation for motility analysis

For motility analysis, samples were divided into aliquots for control and antioxidant test conditions. Controls had no antioxidant added to the preparation medium while test aliquots had all of the preparation media used subsequently for that aliquot (i.e. both layers of Percoll and the BWW medium) supplemented with one of the following antioxidants: (i) GSH (L-γ-glutamyl-L-cysteinylglysine, $C_{10}H_{17}N_3O_6S$; Sigma, Poole, UK) at concentrations of 5, 10 and 20 mM; (ii) hypotaurine (2-aminoethane sulfinic acid; $C_2H_7NO_2S$; Sigma) at concentrations of 5, 10 and 20 mM; (iii) glutathione + hypotaurine (both at concentrations of 5, 10 and 20 mM). Antioxidants were made up in BWW and were present throughout the preparation procedure.

Antioxidant supplementation for analysis of DNA integrity and ROS production

Samples were divided into aliquots for control conditions (no antioxidant present in preparation medium) or test conditions in which one of the following antioxidants had been added to all of the preparation media used subsequently for that aliquot (i.e. both layers of Percoll and the BWW medium): (i) glutathione at a concentration of 10 mM; (ii) hypotaurine at a concentration of 10 mM; (iii) glutathione + hypotaurine (both at concentrations of 10 mM). As before, antioxidants were present throughout the entire preparation procedure.

Division of samples

Of the 45 samples included in the study, 24 were normozoospermic and 21 were asthenozoospermic. These samples were subdivided and each control and antioxidant treatment group consisted of eight normozoospermic and seven asthenozoospermic. In addition, 15 of the samples (five normo- and 10 asthenozoospermic) were subdivided again to give a control, a glutathione-treated aliquot, a hypotaurine-treated aliquot and an aliquot treated with glutathione + hypotaurine all from the one sample. Sperm progressive motility, DNA integrity and ROS production were determined to allow a hierarchical analysis of results in addition to the analysis of pooled groups.

Determination of sperm motility parameters

Sperm motility parameters were analysed immediately after Percoll density centrifugation and at 0.5, 1, 2, 3 and 4 h times. Sperm motility parameters were measured using a Hamilton Thorne Integrated Visual Optical System (IVOS) sperm analyzer (Version 10.7; Hamilton Thorne Research, Beverly, MA). The settings employed for analysis were from: acquisition rate (Hz) 50; minimum contrast 7; minimum size 6; low gate size, 0.4; high gate size, 1.6; low intensity gate, 0.4; high intensity gate, 1.6; magnification factor, 2.04. The motility parameters measured were: rapid progressive motility (those

sperm which exhibit an actual space gain motility); straight line velocity (VSL, the straight line distance from beginning to end of a sperm track divided by the time taken); average path velocity (VAP, the average velocity of sperm movement); curvilinear velocity (VCL, a measure of the total distance travelled by a given sperm divided by the time elapsed); sperm head movements, i.e. the amplitude of lateral head displacement (ALH, the mean width of sperm head oscillation); beat cross frequency (BCF, the frequency of the sperm head crossing the sperm average path). Percentage hyperactivation (HA) was also measured and was defined as VCL > 100, LIN < 65, ALH > 7.5. Cells with VAP > 25 µm/s were counted as exhibiting rapid progressive motility. Motilities were determined for all samples using 20 µm depth Microcell counting chambers (Conception Technologies, La Jolla, CA).

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 (Richardsons Supply Co., London, UK) were covered with 100 µl of 0.5% normal melting point agarose in Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (PBS) (Sigma) at <45°C and immediately covered with a large coverslip. When the agarose had solidified, the coverslips were removed and 1×10^5 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.

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 Na$_2$EDTA, 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 (Sigma) followed by 90 min incubation at 20°C with 4 mM lithium diiodosalicyclate (Sigma) (Robbins *et al.*, 1993). Test slides had the required antioxidant present throughout these lysis and decondensation steps.

Induction of DNA damage using H_2O_2. DNA damage was then induced using H_2O_2 in one control and test slide for each antioxidant. Slides were immersed in a Coplin jar containing 200 µM H_2O_2 (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 Coplin jars 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 mA 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 an excitation filter of 515–560 nm from a 100 W mercury lamp and a barrier filter of 590 nm. Fifty images were captured and analysed by an image analysis system using the programme Komet 3.1.

Measurement of reactive oxygen species (ROS) in sperm

Sperm samples ($n = 10$ each for control, glutathione, hypotaurine and both antioxidants in combination) were prepared by Percoll density centrifugation with and without antioxidant supplementation. ROS were measured with a Lumat LB9507 luminometer (Berthold, Wildbad, Germany) using a modification of the method of Krausz *et al.* (1992). Background activity in a 200 µl aliquot of prepared sperm at a concentration of 1×10^7/ml was measured over a 5 min period. Sperm were then incubated with 25 µM 5-amino-2,3-dihydro-1,4 phthalazinedone (Luminol; Sigma) supplemented 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) (Sigma) in order to stimulate free radical production by leukocytes. The response was recorded for 20 min
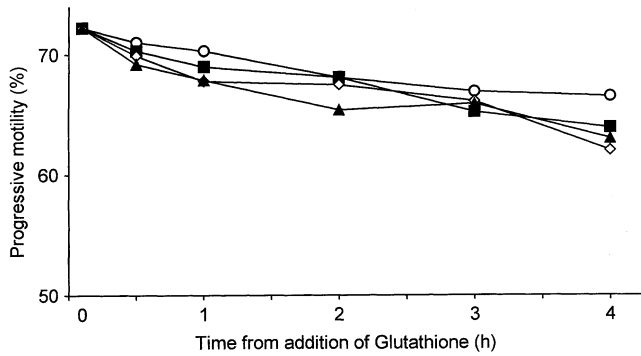
Fig. 1. The effect of glutathione supplementation *in vitro* on progressive motility of normozoospermic sperm samples over a 4 h period showing control values (○) and following incubation with 5 (■), 10 (◇) and 20 mM (▲). Values are medians.
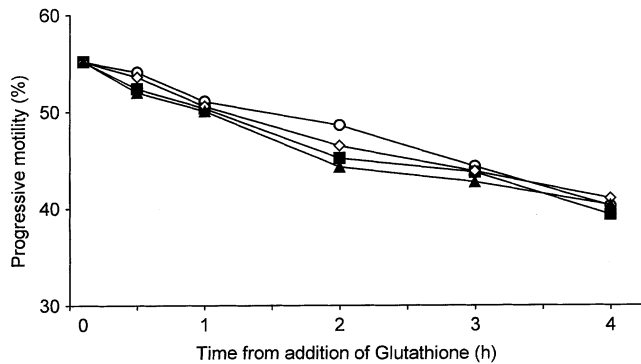

Fig. 2. The effect of glutathione supplementation *in vitro* on progressive motility of asthenozoospermic sperm samples over a 4 h period showing control values (○) and following incubation with 5 (■), 10 (◇) and 20 mM (▲).

Fig. 3. The effect of hypotaurine supplementation *in vitro* on progressive motility of normozoospermic sperm samples over a 4 h period showing control values (○) and following incubation with 5 (■), 10 (◇) and 20 mM (▲). Values are medians.

Fig. 4. The effect of hypotaurine supplementation *in vitro* on progressive motility of asthenozoospermic sperm samples over a 4 h period showing control values (○) and following incubation with 5 (■), 10 (◇) and 20 mM (▲). Values are medians.

to measure the extent of the peak chemiluminescence response which was likely to be attributable to seminal leukocytes and allow the system to settle back to baseline values. The sperm were then stimulated with 100 nm 12-myristate-13-acetate phorbol ester (PMA) (Sigma) 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 glutathione and hypotaurine supplementation on sperm motility, DNA integrity and ROS production.

Results

Effect of glutathione and hypotaurine supplementation in vitro on sperm progressive motility

Supplementation of preparation medium with glutathione at concentrations of 5, 10 and 20 mM had no significant effect on sperm progressive motility or other CASA movement parameters over a 4 h period in both normozoospermic (Figure 1) and asthenozoospermic (Figure 2) samples ($P > 0.05$). Similarly, supplementation with hypotaurine at the same concentrations did not influence sperm motility in either
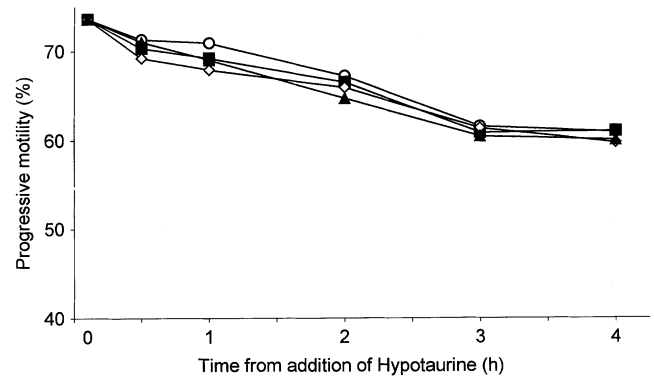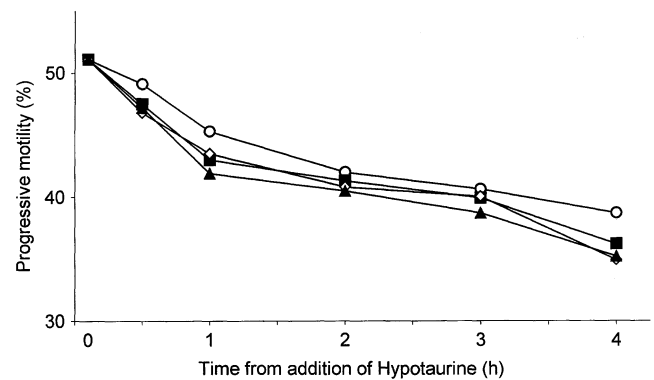
normo- (Figure 3) or asthenozoospermic (Figure 4) samples. Further, no significant effect was recorded when both antioxidants were added in combination (data not shown). Analysis of data showed similar results for both the hierarchical analysis and the pooled groups for both normo- and asthenozoospermic samples.

Effect of glutathione and hypotaurine supplementation in vitro (10 mM) on sperm baseline DNA integrity

DNA integrity of sperm supplemented with glutathione and hypotaurine, either singly or in combination at a concentration of 10 mM, was not significantly different to values observed for controls in both normo- (Figure 5) and asthenozoospermic (Figure 6) samples ($P > 0.05$). Analysis of data showed similar results for both the hierarchical analysis and the pooled groups for both normo- and asthenozoospermic samples. There was no significant difference between sperm baseline DNA integrity for the two different sample groups ($P > 0.05$; Figures 5 and 6).

Effect of glutathione and hypotaurine supplementation in vitro (10 mM) on H_2O_2-induced DNA damage in human sperm

Supplementation with glutathione and/or hypotaurine provided sperm with protection against H_2O_2-induced DNA damage. In normozoospermic samples, percentage intact head DNA was significantly reduced to 58.34% for sperm exposed to 200 μM H_2O_2, compared with 92.65% in untreated controls ($P > 0.005$;
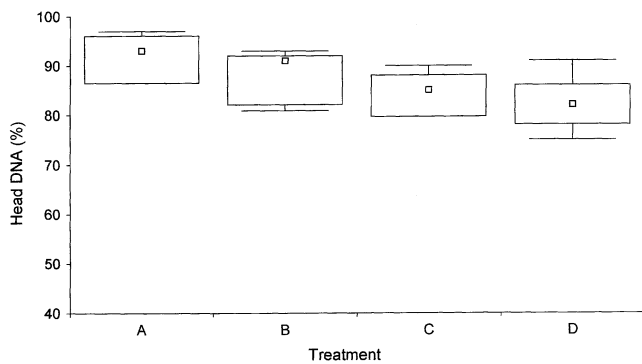
Fig. 5. The effect of supplementation *in vitro* with 10 mM glutathione and 10 mM hypotaurine, both singly and in combination, on sperm DNA integrity in normozoospermic samples. (**A**) Control. (**B**) Sperm treated with 10 mM glutathione. (**C**) Sperm treated with 10 mM hypotaurine. (**D**) Sperm treated with 10 mM glutathione + 10 mM hypotaurine. Values are medians (□) with interquartile ranges (large rectangle, 25–75%) and minimum–maximum values (bars).

Fig. 6. The effect of supplementation *in vitro* with 10 mM glutathione and 10 mM hypotaurine, both singly and in combination, on sperm DNA integrity in asthenozoospermic samples. (**A**) Control. (**B**) Sperm treated with 10 mM glutathione. (**C**) Sperm treated with 10 mM hypotaurine. (**D**) Sperm treated with 10 mM glutathione + 10 mM hypotaurine. Values are medians (□) with interquartile ranges (large rectangle, 25–75%) and minimum–maximum values (bars).
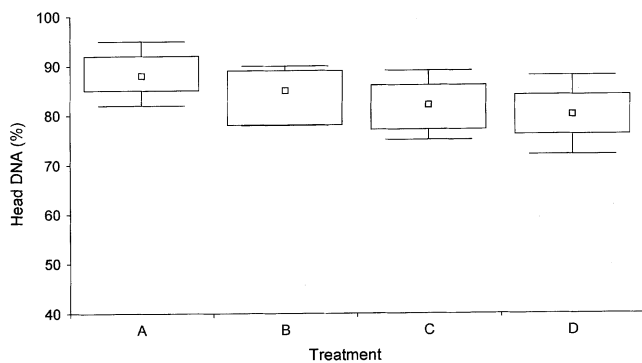
Figure 7). Values of 69.59 and 67.97% intact head DNA were observed for H_2O_2-treated samples supplemented with glutathione or hypotaurine, respectively ($P > 0.01$; Figure 7).

In asthenozoospermic samples, percentage intact head DNA was significantly reduced to 46.88% following exposure to 200 µM H_2O_2, compared with 89.72% in untreated controls ($P > 0.005$; Figure 8). This was a significantly greater reduction in intact DNA than that recorded for normozoospermic samples. Addition of glutathione or hypotaurine to H_2O_2-treated samples increased the intact head DNA to 59.47 and 61.21%, respectively ($P > 0.01$; Figure 8). This percentage increase in undamaged DNA following antioxidant treatment was similar to that observed for normozoospermic samples.

Supplementation *in vitro* of H_2O_2-treated samples with both antioxidants in combination gave 66.05% undamaged DNA in normozoospermic samples and 57.28% undamaged DNA in asthenozoospermic samples ($P > 0.05$). Despite this added protection, results from all antioxidant-treated samples were still significantly lower than untreated controls ($P > 0.01$; Figures 7 and 8). In addition, analysis of the data showed similar results for both the hierarchical analysis and the pooled groups for both normo- and asthenozoospermic samples.
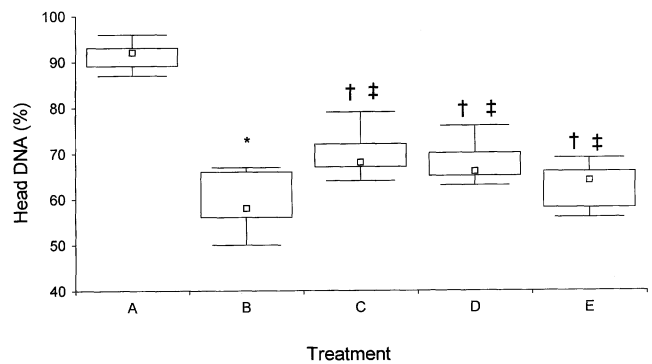

Fig. 7. The effect of supplementation *in vitro* with 10 mM glutathione and 10 mM hypotaurine, both singly and in combination, on sperm DNA integrity in normozoospermic samples treated with 200 µM H_2O_2. (**A**) Control. (**B**) Sperm treated with 200 µM H_2O_2. (**C**) Sperm treated with 10 mM glutathione + 200 µM H_2O_2. (**D**) Sperm treated with 10 mM hypotaurine + 200 µM H_2O_2. (**E**) Sperm treated with 10 mM glutathione + 10 mM hypotaurine + 200 µM H_2O_2. Values are medians (□) with interquartile ranges (large rectangle, 25–75%) and minimum–maximum values (bars). *Significantly lower than control value ($P \leqslant 0.005$, Wilcoxon matched pairs test). †Significantly lower than control value ($P \leqslant 0.01$, Wilcoxon matched pairs test). ‡Significantly greater than value for sperm treated with H_2O_2 only ($P \leqslant 0.005$, Wilcoxon matched pairs test).
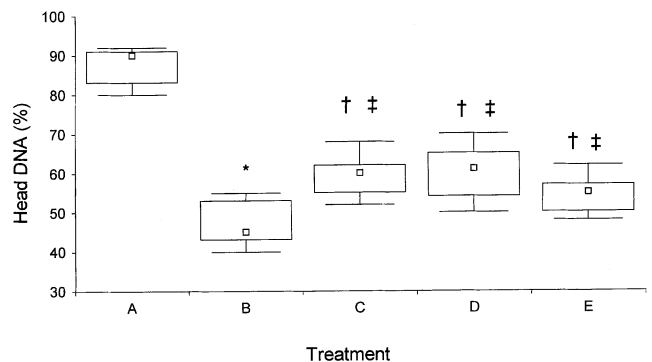

Fig. 8. The effect of supplementation *in vitro* with 10 mM glutathione and 10 mM hypotaurine, both singly and in combination, on sperm DNA integrity in asthenozoospermic samples treated with 200 µM H_2O_2. (**A**) Control. (**B**) Sperm treated with 200 µM H_2O_2. (**C**) Sperm treated with 10 mM glutathione + 200 µM H_2O_2. (**D**) Sperm treated with 10 mM hypotaurine + 200 µM H_2O_2. (**E**) Sperm treated with 10 mM glutathione + 10 mM hypotaurine + 200 µM H_2O_2. Values are medians (□) with interquartile ranges (large rectangle, 25–75%) and minimum–maximum values (bars). *Significantly lower than control value ($P \leqslant 0.005$, Wilcoxon matched pairs test). †Significantly lower than control value ($P \leqslant 0.01$, Wilcoxon matched pairs test). ‡Significantly greater than value for sperm treated with H_2O_2 only ($P \leqslant 0.005$, Wilcoxon matched pairs test).

Effect of glutathione and hypotaurine supplementation in vitro (10 mM) on baseline and H_2O_2-induced free radical production by sperm

Glutathione and hypotaurine, both singly and in combination, at 10 mM did not have any effect on baseline ROS generation by normo- (Figure 9) and asthenozoospermic (Figure 10) samples ($P > 0.05$). However, supplementation with these antioxidants significantly reduced H_2O_2-induced free radical production by sperm in a dose-dependent manner in both sample groups ($P \leqslant 0.005$; Figures 9 and 10). Analysis of data showed similar results for both the hierarchical analysis and the pooled groups for both normo- and asthenozoospermic samples.

Discussion

Reactive oxygen species such as HO·, $O_2^{·-}$ and H_2O_2 are capable of attacking almost all cellular components, including
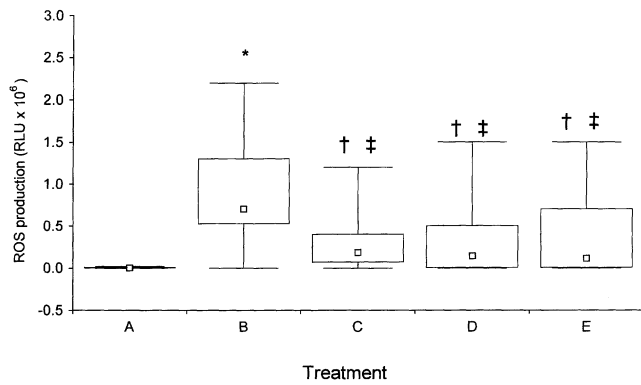
Fig. 9. The effect of supplementation *in vitro* with 10 mM glutathione and 10 mM hypotaurine, both singly and in combination, on ROS production by normozoospermic sperm samples treated with 200 µM H_2O_2. (**A**) Control. (**B**) Sperm treated with 200 µM H_2O_2. (**C**) Sperm treated with 10 mM glutathione + 200 µM H_2O_2. (**D**) Sperm treated with 10 mM hypotaurine + 200 µM H_2O_2. (**E**) Sperm treated with 10 mM glutathione + 10 mM hypotaurine + 200 µM H_2O_2. Values are medians (□) with interquartile ranges (large rectangle, 25–75%) and minimum–maximum values (bars). *Significantly lower than control value ($P \leqslant 0.005$, Wilcoxon matched pairs test). [†]Significantly lower than control value ($P \leqslant 0.01$, Wilcoxon matched pairs test). [‡]Significantly greater than value for sperm treated with H_2O_2 only ($P \leqslant 0.005$, Wilcoxon matched pairs test).
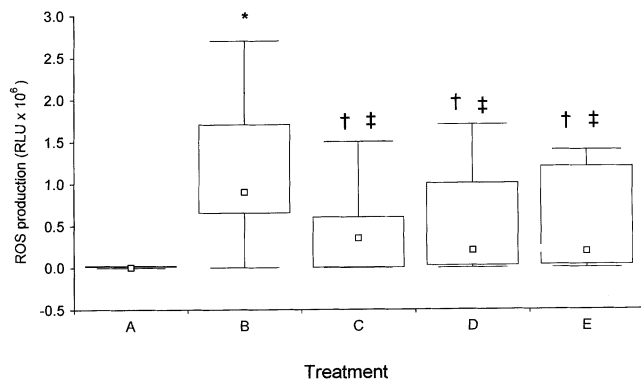

Fig. 10. The effect of supplementation *in vitro* with 10 mM glutathione and 10 mM hypotaurine, both singly and in combination, on ROS production by asthenozoospermic sperm samples treated with 200 µM H_2O_2. (**A**) Control. (**B**) Sperm treated with 200 µM H_2O_2. (**C**) Sperm treated with 10 mM glutathione + 200 µM H_2O_2. (**D**) Sperm treated with 10 mM hypotaurine + 200 µM H_2O_2. (**E**) Sperm treated with 10 mM glutathione + 10 mM hypotaurine + 200 µM H_2O_2. Values are medians (□) with interquartile ranges (large rectangle, 25–75%) and minimum–maximum values (bars). *Significantly lower than control value ($P \leqslant 0.005$, Wilcoxon matched pairs test). [†]Significantly lower than control value ($P \leqslant 0.01$, Wilcoxon matched pairs test). [‡]Significantly greater than value for sperm treated with H_2O_2 only ($P \leqslant 0.005$, Wilcoxon matched pairs test).

DNA, proteins and lipid membranes, and can cause lethal damage to cells (Inoue *et al.*, 1993). The cytotoxic nature of superoxide is most probably mediated by H_2O_2 and its ability to generate the intracellular HO· radical. Both $O_2\cdot^-$ and HO· radicals 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 methyltartronylurea, urea and 5-hydromethyluracil (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 (Demple and Linn, 1982; Breimer and Lindahl, 1985).

Sperm are particularly sensitive to such oxidative attack, especially when they have been prepared for use in assisted conception and have been separated from the protection of their seminal plasma. It has previously been demonstrated that production of ROS is strongly associated with loss of sperm motility (Aitken *et al.*, 1989). Further, an inverse correlation has been observed between the percentage of motile sperm and the quantity of ROS detected in the semen (Iwasaki and Gagnon, 1992). Free radicals such as HO·, $O_2\cdot^-$ and H_2O_2 are known to cause DNA damage in human sperm when exposed for time periods consistent with those used for clinical sperm preparation techniques (Lopes *et al.*, 1998).

Supplementation of sperm preparation media with antioxidants has often been suggested as a way of reducing ROS-induced damage in sperm. Previous studies from our laboratory have found that *in vitro* supplementation with ascorbate and α-tocopherol was not beneficial to sperm motility (Donnelly *et al.*, 1999b) but did provide protection against induced DNA damage and ROS production (Hughes *et al.*, 1998; Donnelly *et al.*, 1999a). The aim of the present study was to extend this work and to examine the effects of other antioxidants, namely glutathione and hypotaurine, on sperm motility, DNA integrity and ROS production.

Glutathione is the most abundant non-protein thiol in mammalian cells (Irvine, 1996). Cellular GSH plays a key role in biological processes, including the synthesis of proteins and DNA and the transport of amino acids. However, its most important role is in protecting cells against oxidation; the sulphydryl group (SH) is a strong nucleophile and confers protection against damage by oxidants, electrophiles and free radicals (Levy *et al.*, 1993). High concentrations of GSH have been observed in rat and mouse testis (Grosshans and Calvin, 1985). A 3-fold increase in the concentration of GSH in rat testis is observed during the onset of spermatogenesis. Isolated spermatocytes and spermatids from hamsters contain large amounts of reduced GSH and are capable of GSH synthesis and of using GSH-dependent defence mechanisms (Den Boer *et al.*, 1989, 1990). While substantial amounts of GSH are located in the testis, reproductive tract fluids and epididymal spermatozoa, much less is present in ejaculated spermatozoa (Agrawi and Vahna-Perttula, 1988).

Thiol-containing molecules such as GSH can exhibit many different kinds of antioxidant activity, serving as sacrificial antioxidants when used in high concentrations. Glutathione is involved in a protective mechanism that involves inactivation of ROS, including peroxides formed in cellular oxygen metabolism. These toxic oxygen species may be detoxified by reduction with GPX, which is converted to oxidized glutathione (GSSG). In turn, GSSG is reduced by glutathione reductase, in the presence of NADPH. Glutathione peroxidase is a selenocysteine-containing antioxidant enzyme that plays a role in the elimination of hydrogen peroxide (Fridovich, 1983) and is also known to be involved in the detoxification of reactive lipids (Chiu *et al.*, 1982).

GSH can neutralize hydroxyl radicals and singlet oxygen. Glutathione could also function in the detoxification of peroxides through its interaction with sperm GPX, although the limited membrane permeability of GSH would reduce the effectiveness of this particular mechanism (Baker *et al.*, 1996). It may also facilitate the antioxidant action of α-tocopherol in

the sperm plasma membrane by participating in the regeneration of this vitamin from tocopheryl radicals. Parenteral administration of GSH in a controlled trial has recently been shown to improve sperm motility and morphology in infertile men with abnormal semen quality associated with varicoceles or sterile genital tract inflammation (Lenzi *et al.*, 1993).

Hypotaurine is a preventative antioxidant that scavenges the hydroxyl radicals involved in the initiation of peroxidative damage. It is known to prevent sperm lipid peroxidation (Alvarez and Storey, 1983). It has been suggested that hypotaurine acts as an antioxidant in sperm cells and is oxidized to taurine during the process (Holmes *et al.*, 1992). The hypotaurine/taurine status in sperm and genital secretions seems to be important for the elucidation of the physiological role(s) of these amino acids.

The exact mechanism of action of hypotaurine is somewhat uncertain, although it appears to display hydroxyl radical scavenging activity (Pasantes-Morales and Fellman, 1989). It is thought that hypotaurine is able to react directly with cytotoxic aldehydes produced during lipid peroxidation and thus protects the thiol groups on the sperm plasma membrane (Lopes *et al.*, 1998).

Hypotaurine concentrations of 35–300 µM have been reported in genital secretions (Guérin and Ménézo, 1995). The antioxidant function of hypotaurine is effective at 10 µM (Aruoma *et al.*, 1988) and minimum effective concentrations of hypotaurine and taurine for the protection of hamster sperm motility are 0.1 and 0.3 µM, respectively (Gwatkin, 1983). Thus, hypotaurine in genital fluids may have protective functions for gametes and embryos. It has been found that after fertilization, taurine and hypotaurine are permanently present in the environment of the gametes and embryos (Guérin and Ménézo, 1995). Hypotaurine and taurine ratios are lower in serum than in tubal and follicular fluids. This implies an active secretory mechanism and suggests the existence of selective transfer of hypotaurine in genital fluids from serum and synthesis and secretion by genital epithelia (Guérin and Ménézo, 1995).

Hypotaurine and taurine are present at very high concentrations in both the male and female reproductive tract fluids (Meizel *et al.*, 1980; Casslen, 1987). In male reproductive tract fluids the concentrations of hypotaurine are modulated by androgens, thus implicating their importance in male reproductive function (Kochakian, 1973).

We have found that supplementation of preparation medium with glutathione and hypotaurine, both singly and in combination, at concentrations of 5, 10 and 20 mM had no significant effect on sperm progressive motility or other CASA movement parameters in both normo- and asthenozoospermic samples. It has previously been shown that GSH protects sperm from ROS-induced loss of motility (Griveau and Lannou, 1994) and incubation for 5 h with 1–10 mM GSH appears to improve sperm motility damaged by activated polymorphonuclear leukocytes (Baker *et al.*, 1996). In addition, hypotaurine has been shown to support hamster sperm motility, capacitation and fertilization *in vitro* (Mrsny *et al.*, 1979; Leibfried and Bavister, 1981). Hypotaurine has also been shown to be effective in protecting human spermatozoa from leukocyte-derived oxidative stress (Baker *et al.*, 1996). It has also been shown to sustain the motility of rabbit sperm by protecting these cells from oxidative damage (Alvarez and Storey, 1983).

Also, in contrast to our results, Parinaud *et al.* (1997) found that treating sperm with GSH-supplemented medium during liquefaction and centrifugation increased the recovery of motile sperm after preparation by Percoll density centrifugation. All samples were leukocytospermic with $>1\times10^6$/ml leukocytes present, suggesting that antioxidants may have a useful role in samples where excessive ROS generation is occurring. However, as samples were divided immediately after ejaculation it is possible that, due to lack of liquefaction, the resulting aliquots were not equal, which may incorporate some bias into the study. Also, the authors do not specify exactly what concentration of GSH was used in the incubation medium, which makes a direct comparison with our study difficult.

Our present study found that supplementation *in vitro* with glutathione and hypotaurine at a concentration of 10 mM had no significant effect on sperm baseline DNA integrity and baseline ROS production in both normo- and asthenozoospermic samples. However, supplementation with these antioxidants did provide sperm from normo- and asthenozoospermic samples with similar levels of protection against H_2O_2-induced DNA damage and ROS production. This is in agreement with earlier studies which found that GSH and hypotaurine (10 mM) significantly reduced DNA fragmentation induced by ROS generation in human sperm using the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) assay (Lopes *et al.*, 1998).

Our results also show that baseline DNA integrity is similar in normo- and asthenozoospermic samples. However, 200 µM H_2O_2 induced a greater amount of DNA damage in asthenozoospermic samples compared with normozoospermic samples. This is in agreement with earlier studies from our laboratory where sperm from fertile men were found to be less susceptible to damage induced by both X-irradiation and H_2O_2 than sperm from infertile men (Hughes *et al.*, 1996). The H_2O_2-induced stress used in the current study may be representative of stress encountered during *in vitro* fertilization procedures. Patients may have experienced a high level of dietary stress prior to semen sampling that may leave sperm exposed to high levels of ROS. There may also be contaminants in preparation media which could create a stressed environment for sperm. In addition, it is likely that sperm preparation techniques which involve the use of repeated centrifugation steps enhance the production of ROS which are detrimental to sperm (Aitken and Clarkson, 1987).

Our results have shown that *in vitro* supplementation with a combination of GSH and hypotaurine was no more powerful at reducing H_2O_2-induced DNA damage and ROS production in either normo- or asthenozoospermic samples than supplementation with each antioxidant in isolation. A combination of glutathione and hypotaurine together has previously been shown to be more effective at protecting sperm DNA from ROS-induced fragmentation than the addition of each antioxidant in isolation (Lopes *et al.*, 1998). In addition, both antioxidants in combination have been shown to be capable of protecting human spermatozoa from leukocyte-derived oxidative stress (Baker *et al.*, 1996), although there was no statistical difference between this combination and the addition of glutathione in isolation.

This study has shown that the addition of glutathione or hypotaurine, both singly and in combination, to sperm preparation media does not significantly improve sperm motility, baseline DNA integrity or baseline ROS production in normo- and asthenozoospermic samples. However, these antioxidants still provide sperm from both these sample groups with protection against subsequent H_2O_2-induced DNA damage

and also cause a significant decrease in H_2O_2-induced ROS production. These results may have clinical applications for the preparation of poor quality sperm for ICSI, which are known to have higher levels of ROS than sperm from fertile individuals. Further research is likely to be required in order to select the best antioxidant or combination of antioxidants that will mimic the action of seminal plasma and provide sperm with extra protection against free radical attack.

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