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OUTSTANDING CONTRIBUTION

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

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Exposure of human spermatozoa to nicotinamide adenine dinucleotide phosphate (NADPH) resulted in the dose dependent generation of reactive oxygen species (ROS) which, at a critical level of intensity, induced lipid peroxidation, DNA damage and a dramatic decline of sperm motility. This system was then used as a model for screening the ability of different antioxidants to combat oxidative stress created through the excessive intracellular generation of toxic oxygen products of metabolism. A variety of antioxidants that has previously been shown to be protective against extracellularly derived oxidants (e.g. superoxide dismutase, catalase, vitamin E, hypotaurine) were ineffective in this system. Albumin, however, could provide complete protection against NADPH induced oxidative stress via mechanisms that did not involve the suppression of the lipid peroxidation cascade but rather the inactivation of lipid peroxides generated during this process. Albumin did not protect against DNA damage induced by NADPH but was extremely effective at preventing DNA fragmentation arising from the suppression of glutathione peroxidase activity with mercaptosuccinate. These studies emphasize that the design of clinically effective antioxidant treatments will depend, critically, upon the source of the oxidative stress. For cases involving excessive intracellular ROS generation, albumin appears to be an important means of neutralizing lipid peroxide-mediated damage to the sperm plasma membrane and DNA.

Key words: albumin/DNA damage/NADPH/reactive oxygen species/sperm motility

Introduction

Oxidative stress is known to play a major role in the aetiology of defective sperm function via mechanisms involving the induction of peroxidative damage to the plasma membrane (Jones *et al.*, 1979; Aitken and Clarkson, 1987; Iwasaki and Gagnon, 1992; Aitken and Fisher, 1994; Sharma and Agarwal, 1996; Griveau and Le Lannou, 1997b). In addition, oxidative stress is known to affect the integrity of the sperm genome, inducing high frequencies of single and double DNA strand breaks (Hughes *et al.*, 1996; Twigg *et al.*, 1997). The clinical significance of such oxidative damage to human spermatozoa has been suggested by the correlations observed between the generation of reactive oxygen species (ROS) by washed human sperm suspensions and the fertilizing capacity of the spermatozoa (Aitken *et al.*, 1991; Krausz *et al.*, 1994; Sharma and Agarwal, 1996; Sukcharoen *et al.*, 1996).

Given the detrimental effect of ROS on human sperm function, antioxidants might be expected to provide a possible route for the treatment of male infertility *in vivo* and *in vitro*. *In vivo*, a recent study of patients with asthenozoospermia in association with peroxidative injury to the spermatozoa has demonstrated the potential value of oral vitamin E therapy in a placebo controlled trial (Suleiman *et al.*, 1996). *In vitro*, a variety of models have been developed for screening the ability of different antioxidants to protect human spermatozoa from oxidative damage. For example, xanthine oxidase has been used to generate a mixture of reactive oxygen metabolites that suppress the motility of human spermatozoa with considerable efficiency. The addition of catalase, but not superoxide dismutase (SOD), to such preparations has been shown to rescue sperm movement, suggesting that hydrogen peroxide rather than superoxide is damaging to human spermatozoa under these circumstances (De Lamirande and Gagnon, 1992; Aitken *et al.*, 1993a; Griveau *et al.*, 1995). In keeping with this conclusion direct exposure of human spermatozoa to hydrogen peroxide has been shown to induce a loss of functional competence (Oehninger *et al.*, 1995) and high rates of DNA damage (Hughes *et al.*, 1996; Twigg *et al.*, 1997). A second strategy that has been used to create oxidative stress in human sperm populations involves co-incubation of these cells with activated neutrophils (Baker *et al.*, 1996). This treatment leads to a significant loss of sperm motility that can be rescued by certain antioxidants, such as hypotaurine and N-acetylcysteine, but not others, such as vitamin E (Baker *et al.*, 1996).

The major problem with all of the approaches outlined above is that the ROS that attack the spermatozoa originate from outside these cells. This situation is physiologically meaningful in the context of assessing antioxidants for their ability to intercept the toxic oxygen metabolites generated by infiltrating leukocytes (Baker *et al.*, 1996). However, the source of cytotoxic oxygen radicals is frequently intracellular, as in the case of oligozoospermic males whose spermatozoa generate particularly high levels of ROS (Aitken *et al.*, 1992a; Gomez *et al.*, 1996). In order to examine alternative antioxidant

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strategies and identify those that best protect spermatozoa from the excessive generation of intracellular ROS, a different model system is needed that involves the accelerated generation of toxic oxygen metabolites by the spermatozoa themselves. The recent observation (Aitken *et al.*, 1997) that ROS production by human spermatozoa is dependent on NADPH and can be artificially stimulated by exposure to this reagent, suggests a basis for just such a model. In this paper we have examined the influence of NADPH-induced ROS generation on the functional and genomic integrity of human spermatozoa and then used this system to examine the ability of different antioxidant strategies to provide protection against the excessive intracellular generation of toxic oxygen metabolites.

Materials and methods

Human sperm preparation

This study was based upon semen samples donated by a panel of healthy, normozoospermic donors (World Health Organization, 1992) after 2 or 3 days' abstinence. The samples were produced into sterile containers and left for at least 30 min to liquefy before being processed by discontinuous Percoll gradient centrifugation (Aitken *et al.*, 1996a). The spermatozoa recovered from the high density region of the gradient were suspended at a final concentration of 20×10^6/ml in HEPES-buffered Biggers–Whitten–Whittingham medium (BWW) (Biggers *et al.*, 1971) supplemented with 1 mg/ml polyvinyl alcohol (PVA) or 0.3% bovine serum albumin (BSA; fraction V, nuclease and protease free, Calbiochem, Nottingham, UK), where indicated. All of the preparations used in this study to examine the impact of NADPH on ROS generation and sperm function were polymorph-free in that they had been screened with a formyl methionyl leucyl phenylalanine (FMLP; Sigma, Poole, UK) provocation test (Krausz *et al.*, 1992) and, if a positive signal was obtained, subsequently incubated with magnetic beads coated with an anti-CD45 monoclonal antibody (Aitken *et al.*, 1996b). Following this treatment the samples were tested again with the FMLP provocation test and only if a negative response was obtained were they carried forward for analysis.

Motility

Sperm motility was monitored throughout the experiments using methodology described by the World Health Organisation (1992). For these assessments, 10 μl of semen was placed on a microscope slide pre-warmed to 37°C and covered with a 19×19 mm coverslip. The preparation was then examined at ×100 magnification using phase-contrast optics and counted with the aid of a grid on an eyepiece graticule. Spermatozoa in WHO categories *a*, *b* and *c* were regarded as motile. For each incubation the motility of the cells was monitored at the beginning of the experiment and after an incubation period of 24 h; the percentage motility loss was then calculated.

Assessment of DNA damage

The assessment of DNA damage involved a preliminary decondensation of the spermatozoa using a protocol similar to that described by West *et al.* (1989), before performing an in-situ nick translation assay for the detection of DNA strand breaks (Twigg *et al.*, 1997). For these analyses, a 500 μl aliquot of each sample was centrifuged at 500 *g* for 5 min, resuspended in 1 ml of BWW containing 6 mM ethylenediaminetetraacetic acid (EDTA; BDH, Poole, UK) and centrifuged again at 500 *g* for 5 min. One ml of BWW containing 2 mM dithiothreitol (DTT; Calbiochem) was then added to the pellet and the spermatozoa incubated for a further 1 h at 37°C under 95% air and 5% CO_2. Once the incubation in DTT had been completed the spermatozoa were centrifuged at 500 *g* for 5 min and then fixed with the correct volume of a 3:1 ethanol:glacial acetic acid mixture (Analar grade, BDH) to bring their concentration back to 10×10^6/ml. Ten μl of this suspension was dropped onto a demarcated area on a clean glass microscope slide and allowed to air dry. A small volume of BWW containing 0.05% sodium dodecyl sulphate (SDS; Promega, Southampton, UK) was then added to each slide and allowed to stand for 2 min. Each slide was then rinsed in tap water followed by two washes in phosphate buffered saline (PBS; Sigma).

In order to block any endogenous biotin/avidin binding sites within the spermatozoa (Wood and Warnke, 1981) a blocking step was introduced comprising sequential 20 min incubations with 0.001% biotin (Sigma) and 0.01% avidin (Sigma) in distilled water. In-situ nick translation was then performed using DNA polymerase 1 (Promega) and biotin 16-dUTP (Boehringer Mannheim, Lewes, UK) (Twigg *et al.*, 1997). Biotin incorporation was detected by an alkaline phosphatase; 5-bromo-4-chloro-3-indoyl phosphate: nitroblue tetrazolium (NBT; Boehringer Mannheim) method (De Jong *et al.*, 1985). Following staining, 50 randomly selected spermatozoa were captured at ×400 magnification on an Olympus BH2® microscope and scored for optical density using an IBM compatible PC running Image Pro Plus® for Windows v1.3.2®. Data were saved for analysis on an Excel® spreadsheet.

Chemiluminescent measurement of ROS

ROS generation was monitored with lucigenin-dependent chemiluminescence on a Berthold LB9505 luminometer (Aitken *et al.*, 1992b). For this assay 400 μl of sperm suspension was placed in a sample tube and mixed with 4 μl of a 25 mM lucigenin stock solution dissolved in dimethylsulphoxide. The chemiluminescent signal was then monitored and, once it had stabilized, the reagent of interest was added and the counts integrated over a 5 min period.

Lipid peroxidation

Lipid peroxidation was monitored using the malondialdehyde assay as described by Aitken *et al.* (1993b). In brief, the spermatozoa were centrifuged at 500 *g* for 5 min and then resuspended in Hanks' balanced salt solution lacking Ca^{2+} and Mg^{2+} but supplemented with Fe^{2+} and ascorbate, to promote a lipid peroxidation cascade resulting in the generation of malondiadehyde. Standards were created by the acid hydrolysis of 1,1,3,3,-tetraethoxypropane by overnight incubation in the presence of 0.1 M HCl.

Statistics

The data were analysed using the Statview® (Abacus Concepts, Berkeley, CA, USA) software programme on an Apple® Macintosh computer. Each experiment was replicated at least three times and the significance of the results examined by one way analysis of variance (ANOVA) for repeated measures. Post-hoc testing of differences between groups was examined by Fisher's PLSD (protected least significant difference) with the significance threshold set at $P < 0.05$. Paired comparisons were conducted using both the paired F and the Wilcoxon sign rank test.

Results

NADPH dose response analysis

A dose dependent analysis was conducted of the ability of NADPH to stimulate ROS production, induce peroxidative damage and disrupt sperm motility. The doses studied were 1,

5 and 10 mM NADPH and, as controls, equivalent doses of $NADP^+$. The incubations were conducted in medium BWW supplemented with 1 mg/ml PVA and the duration of the incubation was 24 h. The results, presented in Figure 1, reveal that incubation with 1, 5 or 10 mM $NADP^+$ had no significant effect on the generation of ROS by the spermatozoa, the level of peroxidative damage sustained by these cells or their motility over a 24 h period. In contrast, all three doses of NADPH induced significant increases in ROS production ($P < 0.001$; Figure 1, Panel A) and, at the two highest doses tested, the level of ROS generation was adequate to induce significant increases in lipid peroxidation (Figure 1, Panel B) and highly significant reductions in sperm motility (Figure 1, Panel C; $P < 0.001$).

Influence of SOD and catalase

In view of the beneficial effects that have been observed with SOD and catalase in preserving human sperm function (Aitken *et al.*, 1993a; Griveau and Le Lannou, 1994), experiments were conducted to assess the ability of these antioxidant enzymes to suppress the peroxidative damage induced by endogenously generated ROS. Catalase (200, 500 and 2000 units) and SOD (100, 250 and 500 units) were added to the sperm suspensions and incubated for 24 h in the presence or absence of 5 mM NADPH. NADPH again induced a highly significant loss of sperm motility (Figure 2, Panel A; $P < 0.001$) in concert with a highly significant stimulation of peroxidative damage to the spermatozoa (Figure 2, Panel B; $P < 0.001$). However, none of the doses of SOD or catalase, alone or in combination, had a significant effect on either the maintenance of sperm motility or the amount of peroxidative damage sustained by these cells (Figure 2, Panels A and B).

Free radical scavengers

A variety of free radical scavengers were used to determine whether they could suppress the peroxidative damage and concomitant motility loss observed following the induction of ROS generation by NADPH. None of the following antioxidants were found to be effective in this regard: vitamin E (1 mM), butylated hydroxytoluene (0.01, 0.1 and 1.0 mM), pentoxifylline (3.6 mM) and hypotaurine (1.0 mM; Figure 3, Panel A). In addition, the iron chelator diethylenetriamine-pentaacetic acid (DETAPAC, 0.2–2.0 mM; Figure, Panel B) could not protect human spermatozoa from the oxidative damage induced by NADPH. However, DETAPAC did significantly ($P < 0.05$) enhance the preservation of motility in the control incubations, suggesting that iron-induced lipid peroxidation contributes to the spontaneous loss of motility observed following the prolonged incubation of human spermatozoa *in vitro* (Figure 3, Panel B).

Albumin

In an additional series of experiments the importance of albumin in protecting human spermatozoa from endogenously generated oxidative stress was examined. The experimental design involved exposing human spermatozoa to NADPH in the presence of PVA (1 mg/ml) or a range of albumin concentrations (0.3, 1.0, 3.0 and 10.0%) and after 24 h at 37°C

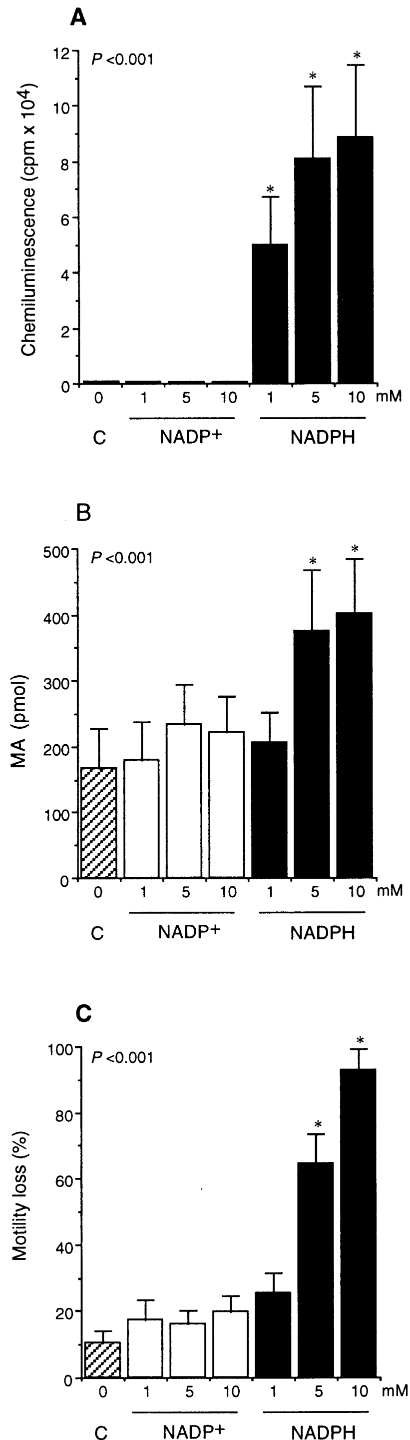

Figure 1. Dose-dependent analysis of the impact of NADPH and $NADP^+$ on human spermatozoa. (**A**) the generation of ROS; (**B**) the induction of lipid peroxidation expressed as pmol malondialdehyde (MA) generated by 2×10^6 spermatozoa in 2 h and (**C**) the percentage loss of motility. C = control medium. ANOVA revealed a highly significant effect of treatment ($P < 0.001$) for all three variables. *Post-hoc testing with Fisher's PLSD for the differences between NADPH and control with the significance level set at $P < 0.05$; $n = 6$.

assessing the motility and lipid peroxidation potential of the spermatozoa. In the presence of PVA the induction of peroxidative damage by NADPH led to a dramatic loss of

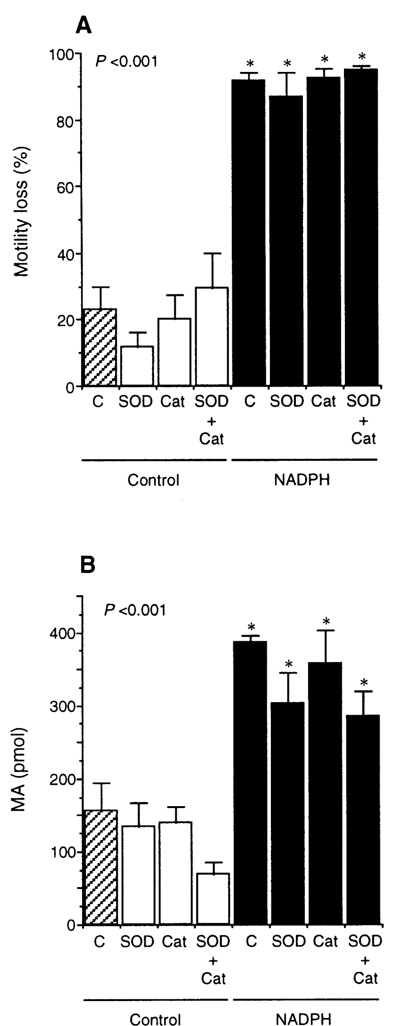

Figure 2. Analysis of the influence of SOD (250 U) and catalase (500 U) on the induction of lipid peroxidation and motility loss observed on exposure of human spermatozoa to NADPH. **A**, percentage motility loss; **B**, lipid peroxidation expressed as pmol malondialdehyde (MA) generated by 2×10^6 spermatozoa in 2 h. **C** = control medium; Cat = catalase. ANOVA revealed a highly significant effect of treatment ($P < 0.001$) for both variables. *Post-hoc testing with Fisher's PLSD for the differences between NADPH and control with the significance level set at $P < 0.05$; $n = 5$.

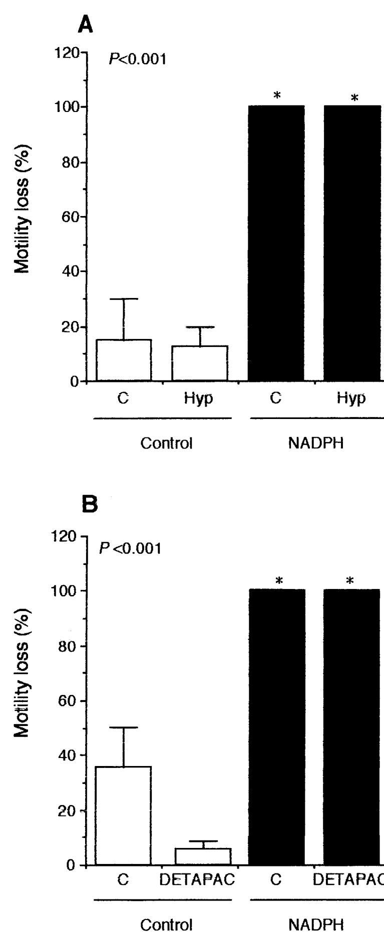

Figure 3. Influence of different antioxidant strategies on the induction of motility loss by NADPH. (**A**) = hypotaurine (Hyp) and (**B**) = DETAPAC. ANOVA revealed a highly significant effect of treatment ($P < 0.001$) for both compounds. *Post-hoc testing with Fisher's PLSD for the differences between NADPH and control with the significance level set at $P < 0.05$; $n = 3$.

sperm motility as observed in previous experiments (Figure 4, Panel A). In the absence of NADPH, the presence of albumin in the incubation medium had no significant effect on sperm motility relative to the PVA controls (Figure 4, Panel A). However in the presence of NADPH, a very profound effect of albumin supplementation was observed, with sperm motility being effectively preserved over a range of albumin concentrations from 0.3–10% (Figure 4, Panel A). Since high concentrations of BSA were not found to interfere with the ability of human spermatozoa to generate ROS (data not shown), the influence of albumin on the levels of lipid peroxidation recorded in the spermatozoa was examined. Surprisingly, the levels of malondiadehyde generated by the spermatozoa in the presence of albumin were no different from those recorded in the presence of PVA (Figure 4, Panel B). This result suggested that the presence of albumin did not suppress lipid peroxidation, or even remove lipid peroxides from the spermatozoa, during the stimulation of ROS production with NADPH. Instead, the presence of albumin appeared to neutralize the impact of oxidative stress on the spermatozoa, by binding to toxic lipid peroxides generated within the plasma membrane during the lipid peroxidation cascade. Experiments were subsequently undertaken to examine this hypothesis.

In the first phase of this experiment it had been demonstrated that when lipid peroxidation is promoted with iron and ascorbate, a majority of the peroxides generated during the lipid peroxidation cascade were released into the ambient medium and not retained by the spermatozoa (Figure 5). Thus after 24 h incubation in the presence of 5 mM NADPH the spermatozoa were pelleted by centrifugation (500 *g* for 5 min) and resuspended in the presence of a ferrous ion promoter (0.04 mM $FeSO^4$ and 0.2 mM ascorbate in Ca^{2+} and Mg^{2+}-free Hanks') for 2 h. Unpromoted samples were incubated in Ca^{2+} and Mg^{2+}-free Hanks' medium lacking promoter. At the end of the promotion period malondialdehyde was measured in the whole sperm suspension, the isolated spermatozoa and the incubation medium. This revealed that around 70% of the malondiadehyde generated in the presence of promoter is released from the cells into the surrounding medium (Figure 5).

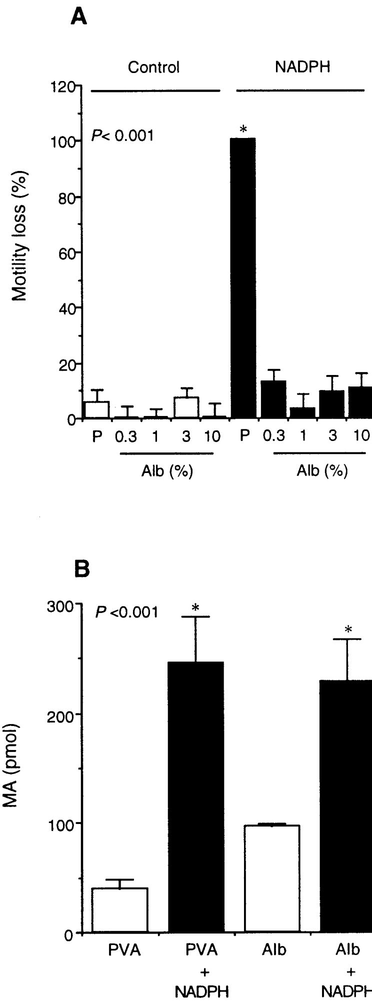

Figure 4. Influence of a dose response for albumin on motility loss and lipid peroxidation induced in human spermatozoa on exposure to NADPH. (**A**) motility loss; (**B**) lipid peroxidation expressed as pmol malondialdehyde (MA) generated by 2×10^6 spermatozoa in 2 h. Alb = albumin; P = PVA. ANOVA revealed a highly significant effect of treatment ($P < 0.001$) for both variables. *Post-hoc testing with Fisher's PLSD for the differences between NADPH and control with the significance level set at $P < 0.05$; $n = 4$.

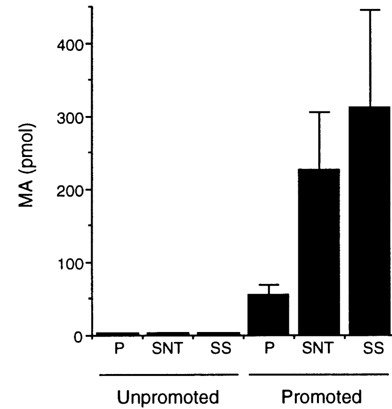

Figure 5. Incubation of human spermatozoa in the presence of a ferrous ion promoter led to the generation of cytotoxic lipid peroxides, a majority of which were released into the ambient medium. Lipid peroxidation expressed as pmol malondialdehyde (MA) generated by 2×10^6 spermatozoa in 2 h. SS = sperm suspension; SNT = supernatant and P = pelleted spermatozoa; $n = 3$.

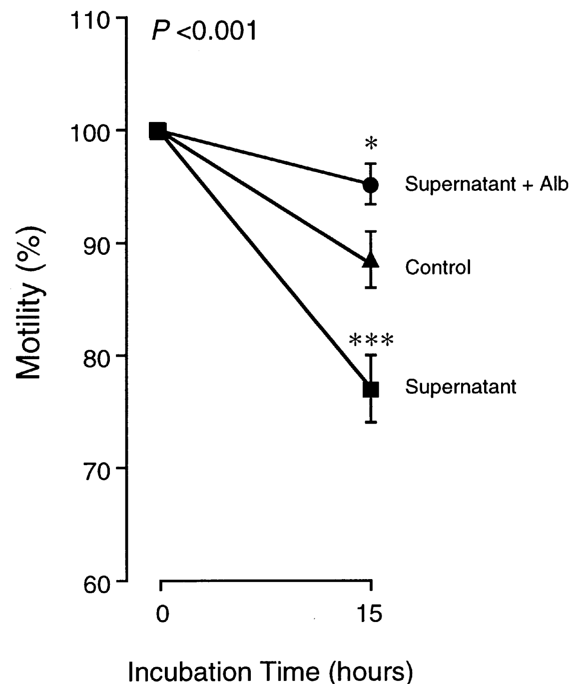

Figure 6. Demonstration of the capacity of albumin to neutralize the cytotoxic effects of lipid peroxides released from spermatozoa in the presence of a ferrous ion promoter. Starting motilities normalized to 100%. *$P < 0.05$, ***$P < 0.001$ by paired *t*- and Wilcoxon sign rank tests; $n = 34$.

The cytotoxicity of the lipid peroxides released from the spermatozoa (Figure 5) was investigated and the protective effect of albumin examined. To achieve this end, medium was recovered from spermatozoa following a 2 h incubation with a ferrous ion promoter (conditioned medium), added to a population of freshly prepared cells and incubated for 15 h *in vitro*. The loss of motility observed over this period of time was compared with cells incubated in the presence of unconditioned promoter. Incubation in the peroxide conditioned medium was found to induce a significant inhibition of motility relative to the controls (Figure 6; $P < 0.001$). In order to see if albumin could negate the effect of peroxides generated by iron promoted peroxidation, an albumin concentration (3%) within the range known to protect sperm motility (Figure 4) was chosen. When added to the conditioned medium at the start of the 15 h incubation not only was the toxic effect of the peroxides neutralized but sperm motility showed a slight enhancement over unconditioned-promoter controls (Figure 6; $P < 0.05$).

DNA damage

In order to determine whether the endogenous generation of ROS could also induce DNA damage in suspensions of human spermatozoa, cells that had been stimulated with NADPH were examined to determine their genomic integrity. The results of this analysis are presented in Figure 7. The induction of ROS generation with NADPH in PVA supplemented medium was associated with the induction of significant DNA damage in

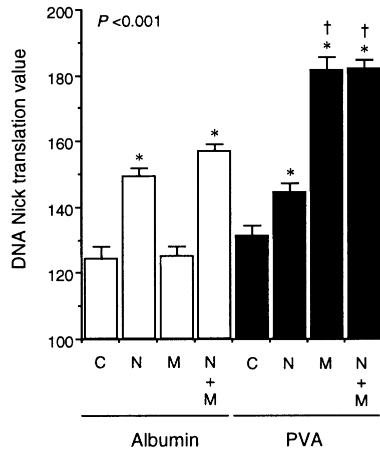

Figure 7. Evaluation of DNA damage in human spermatozoa by nick translation. Ordinate axis gives the intensity of the NBT product as measured by image analysis. C = control; N = NADPH (5 mM), M = mercaptosuccinate (2.0 mM). ANOVA revealed a highly significant effect of treatment ($P < 0.001$). Post-hoc testing with Fisher's PLSD with the significance level set at $P < 0.05$, * identifies differences between treated and respective controls and † identifies differences between PVA and albumin supplemented groups. Each column represents measurements made on 150 cells from three separate individuals.

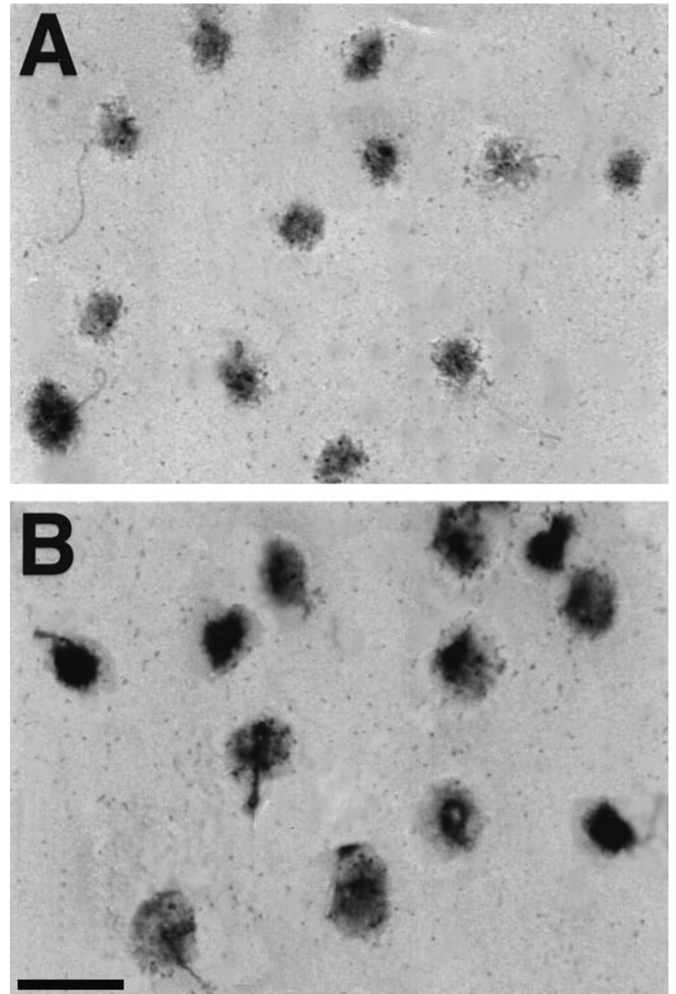

Figure 8. Micrograph demonstrating the high levels of DNA damage induced by mercaptosuccinate. (**A**) mercaptosuccinate in the presence of albumin; (**B**) very high levels of DNA damage induced by mercaptosuccinate in the presence of PVA. The nick translation protocol results in the formation of a black deposit on spermatozoa where DNA strand breaks have been detected. Bar = 50 μm.

the spermatozoa ($P < 0.001$; Figure 7). This damage was markedly enhanced ($P < 0.05$) by the inclusion of mercaptosuccinate (2.0 mM) to suppress glutathione peroxidase (GPX) activity, even under control conditions where ROS production was not stimulated by NADPH (Figures 7 and 8). The damage induced by mercaptosuccinate in the presence of PVA was so great that the addition of NADPH could not elevate the level of DNA fragmentation further (Figures 7 and 8). The presence of albumin, at a concentration routinely used in culture media (0.3%), did not have any significant influence on the basal levels of DNA damage or the increased damage recorded following NADPH addition. However the presence of albumin did have a highly significant beneficial effect on the high levels of DNA damage induced by the presence of mercaptosuccinate in the presence or absence of NADPH (Figure 7).

Discussion

Oxidative stress is an important cause of defective sperm function that should be amenable to antioxidant therapy *in vivo* and *in vitro*. *In vivo*, a recent placebo controlled trial focusing on patients exhibiting high levels of peroxidative damage to their spermatozoa has suggested that oral vitamin E therapy might have therapeutic potential (Suleiman *et al.*, 1996). *In vitro*, studies involving the creation of oxidative stress with activated leukocytes have demonstrated the potential clinical value of a wide range of antioxidants including hypotaurine, reduced glutathione and N-acetylcysteine in protecting human spermatozoa from exogenously-derived, toxic oxygen metabolites (Baker *et al.*, 1996). However, to date, no model has been developed for assessing the ability of different antioxidants to protect human spermatozoa from endogenously generated ROS (Gomez *et al.*, 1996).

The biochemistry of ROS generation by mammalian spermatozoa has not yet been resolved in detail although evidence has recently been obtained indicating that NADPH can serve as a substrate for superoxide generation by these cells (Aitken *et al.*, 1997; Griveau and Le Lannou, 1997a). In this study we have demonstrated that exogenous NADPH is capable of inducing a dose-dependent increase in ROS generation by human spermatozoa that, at the highest doses tested, stimulated significant peroxidative damage and a dramatic loss of sperm motility (Figure 1). None of the reagents that have been shown to protect spermatozoa from exogenously derived ROS including SOD, catalase, hypotaurine, DETAPAC, pentoxifylline or vitamin E, proved effective against NADPH-induced oxidative stress presumably because, on this occasion, the ROS originated intracellularly.

The only supplement that protected spermatozoa from NADPH-induced damage was albumin. In concentrations from 0.3–10%, albumin was able to sustain the motility of human spermatozoa despite the stimulation of high rates of ROS production by NADPH. Moreover, albumin did not appear to

exert its protective action through the inhibition of lipid peroxidation because NADPH induced similar levels of malondialdehyde formation in the presence of albumin or PVA (Figure 4). It is concluded that exposure to NADPH in the presence of either supplement led to the induction of peroxidative damage and an accumulation of lipid hydroperoxides in the plasma membrane. In the presence of PVA these lipid peroxides exerted a cytotoxic effect on the spermatozoa leading to a significant loss of sperm motility. However, in the presence of albumin these lipid peroxides were sequestered in such a way that sperm function was unaffected.

Experimental verification of the ability of albumin to neutralize cytotoxic lipid peroxides was achieved in studies employing a ferrous ion promoter. When the latter was added to spermatozoa, a rapid lipid peroxidation cascade was induced resulting in the release of significant quantities of lipid peroxide into the ambient medium (Figure 5). This peroxide release is presumably a consequence of phospholipase A_2 action, since this enzyme is known to play a protective role in human spermatozoa, cleaving oxidized fatty acids from the 2-position of the phospholipid glycerol backbone so that they can then be metabolized by glutathione peroxidase to the corresponding alcohols (Halliwell and Gutteridge, 1989; Alvarez and Storey, 1995). The presence of such high concentrations of lipid peroxide in the culture medium was undoubtedly a consequence of the presence of a promoter system that would have induced the secondary peroxidation of any unsaturated fatty acids released from the plasma membrane by phospholipase A_2 (Alvarez and Storey, 1995). The peroxides generated were then shown to be cytotoxic in bioassays involving the incubation of freshly prepared spermatozoa in medium conditioned with the lipids released from human spermatozoa in the presence of a ferrous ion promoter. These findings are in keeping with an abundance of evidence indicating that lipid peroxides are cytotoxic to human spermatozoa (Jones *et al.*, 1979). Significantly, the cytotoxic effect of these lipid peroxides could be completely neutralized by the presence of albumin (Figure 6). This result supports the hypothesis that albumin is able to protect the sperm plasma membrane from peroxidative damage by binding to lipid peroxides and neutralizing their cytotoxic activity (Alvarez and Storey, 1995). The mechanisms by which this neutralization is achieved could involve one or more of several different antioxidant properties exhibited by this protein. Albumin not only binds lipid peroxides liberated by phospholipase A_2 but can also impair the lipid peroxidation cascade, either by acting as a sacrificial antioxidant or as a chelator of the transition metals that promote this process (Halliwell and Gutteridge, 1989). Another intriguing possibility is that albumin can exhibit peroxidase activity, converting lipid hydroperoxides to the corresponding alcohols providing that thiol-reducing equivalents, such as reduced glutathione, are available (Cha and Kim, 1996). Albumin may also act by scavenging other cytotoxic products such as peroxynitrite (Gatti *et al.*, 1994) formed by the reaction of superoxide anion with another product of the human spermatozoon, nitric oxide (Donnelly *et al.*, 1997).

The stimulation of endogenous ROS generation with NADPH not only induced peroxidative damage to the sperm plasma membrane but also resulted in a significant increase in the levels of DNA damage sustained by these cells. This increase in DNA damage following exposure to NADPH was evident in the presence of both PVA and albumin. The inability of albumin to suppress NADPH-induced DNA damage could indicate that the causative oxygen metabolite in this context is hydrogen peroxide rather than oxidized lipid (Hughes *et al.*, 1996; Twigg *et al.*, 1997) since albumin does not inhibit the ability of hydrogen peroxide to induce DNA strand breaks (Kozumbo *et al.*, 1996). However, albumin did have a dramatic inhibitory effect on the ability of mercaptosuccinate, which increases lipid peroxidation in human spermatozoa by antagonism of glutathione peroxidase, to induce DNA damage (Chaudiere *et al.*, 1984; Alvarez and Storey, 1989). This observation may be explained by the fact that, in the resting cell, glutathione peroxidase actively scavenges lipid peroxides released from the plasma membrane by phospholipase A_2, in order to protect the spermatozoon's DNA from oxidative attack. Suppression of glutathione peroxidase activity with mercaptosuccinate would then allow these constitutively generated peroxides unrestricted passage from the sperm plasma membrane to the chromatin where they could induce DNA strand breakage. The DNA damage induced by the combination of PVA and mercaptosuccinate appeared to be so great that the stimulation of additional oxidative stress with NADPH was unable to induce further DNA fragmentation (Figure 8). However, in the presence of albumin these membrane derived peroxides were apparently sequestered in such a way that they could no longer translocate to the sperm nucleus and damage the DNA, even if glutathione peroxidase was inactivated with mercaptosuccinate. The following observations are consistent with this hypothesis: (i) lipid peroxides are spontaneously generated in the sperm plasma membrane and are released by the action of phospholipase A_2 (Alvarez and Storey, 1995); (ii) lipid peroxides are capable of inducing DNA damage (Koh *et al.*, 1997); (iii) a form of glutathione peroxidase that is active against phospholipid hydroperoxides is present in mature spermatozoa (Roveri *et al.*, 1992; Alvarez and Storey, 1995; Godeas *et al.*, 1996) and (iv) in spermatozoa, glutathione peroxidase is tightly associated with the nucleus (Roveri *et al.*, 1992; Godeas *et al.*, 1996).

In summary, the results presented in this paper indicate the potential value of utilizing exogenous NADPH to create a model for sperm dysfunction involving the excessive intracellular generation of toxic oxygen metabolites. When the source of ROS is intracellular, many of the classical antioxidants that are perfectly effective against extracellular oxidative attack, such as SOD, catalase or hypotaurine, are ineffectual. However albumin, in doses from 0.3–10%, exhibits an excellent ability to sustain sperm motility in the face of intracellularly generated oxidative stress. Albumin does not prevent lipid peroxidation but binds the lipid peroxides in such a way as to neutralize their cytotoxic action. This protein supplement is also able to protect sperm DNA from oxidative damage generated by the suppression of glutathione peroxidase activity with mercaptosuccinate. However, it cannot prevent the DNA damage induced by NADPH. These studies indicate both the importance and the limitations of albumin as an antioxidant for the protection

of human sperm function and emphasize that the nature of the protein supplements employed in assisted conception protocols should be considered with care.

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