Reactive oxygen species: potential cause for DNA fragmentation in human spermatozoa

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The objective of this study was to evaluate the effect of the generation of reactive oxygen species (ROS) on the integrity of the DNA of human spermatozoa, and to determine if pretreatment with antioxidants can reduce DNA damage. Samples were obtained from 47 men undergoing infertility investigation. ROS were generated in the samples by the addition of xanthine/xanthine oxidase (X/XO) with or without antioxidants. After incubation at timed intervals (0–2 h) with X/XO, the percentage of spermatozoa with DNA fragmentation was determined using the method of TdT-mediated DNA end-labelling (TUNEL). Time intervals were selected to mimic the clinical situation in which spermatozoa are held for a period of time after swim-up while the oocytes are prepared for ICSI. A significant increase in sperm DNA damage was evident when samples were incubated in the presence of ROS for intervals of 1 and 2 h, but not when incubated with ROS for <1 h ($P = 0.0001$). The addition of antioxidants significantly decreased the amount of DNA damage induced by ROS generation ($P < 0.04$). ROS can cause an increase in DNA fragmentation and pretreatment with antioxidants can reduce DNA damage.
Key words: antioxidants/fertilization/male infertility/reactive oxygen species/sperm chromatin

Introduction

Over the past decade, studies have demonstrated that peroxidative damage to the sperm plasma membrane by reactive oxygen species (ROS) may impair sperm function, leading to the onset of male infertility (Aitken *et al.*, 1987; Iwasaki and Gagnon, 1992). ROS include free radicals, which are active oxidizing agents. Spermatozoa are highly susceptible to oxidative damage due to an abundance of polyunsaturated fatty acids within the plasma membrane (Jones *et al.*, 1979) and a low concentration of scavenging enzymes within the cytoplasm. The primary source of ROS in seminal plasma is derived from spermatozoa themselves and from polymorphonuclear leukocytes. The production of ROS by spermatozoa is a normal physiological process required for the occurrence of capacitation and the acrosome reaction (de Lamirande and Gagnon, 1993; de Lamirande *et al.*, 1993). However, high levels of ROS present in seminal plasma have been associated with poor morphology, motility and low sperm count (Aitken *et al.*, 1989; Mazzilli *et al.*, 1994).

We have recently established that poor-quality semen samples have a greater percentage of spermatozoa with DNA fragmentation than normal fertile samples (Sun *et al.*, 1997; Lopes *et al.*, 1998). In addition, we demonstrated a negative correlation between the percentage of spermatozoa with fragmented DNA and fertilization rates in in-vitro fertilization (IVF) (Sun *et al.*, 1997) and in intracytoplasmic sperm injection (ICSI) (Lopes *et al.*, 1998). At present the aetiology of sperm DNA fragmentation in infertile men is unclear. We hypothesize that ROS may be involved in the DNA fragmentation seen in these germ cells since several studies have shown that ROS can cause DNA damage in somatic cells (Buttke *et al.*, 1994; Ratan *et al.*, 1994). The objective of the present study was to evaluate the effect of exogenously generated ROS on the integrity of the DNA of human spermatozoa. We demonstrate that ROS can cause an almost 4-fold increase in DNA fragmentation in a clinically relevant time frame and that pretreatment with several antioxidants can reduce DNA damage.

Materials and methods

Sample collection

A total of 54 semen samples was collected from 47 men undergoing semen analysis in the andrology clinic at The Toronto Hospital, General Division. Written consent for use of the spermatozoa for research was obtained from the patients according to guidelines established for research on human subjects by the University of Toronto.

Semen preparation

Semen samples were collected after at least 48 h of abstinence. After 30 min of liquefaction at room temperature, both routine semen analysis and swim-up assessment were performed using standard techniques. For swim-up, the sample was diluted with human tubal fluid (HTF, Irvine Scientific, Santa Ana, CA, USA) supplemented with 5% human serum albumin (HSA; Irvine Scientific). The diluted semen was washed twice by centrifugation for 10 min at 220 *g*; the final pellet was resuspended in ~200 μl of medium and layered gently under 1 ml of fresh medium supplemented with 5% HSA (Irvine Scientific). The motile spermatozoa were allowed to swim-up for 1 h into the overlaying medium at 37°C in a 5% CO_2 incubator. The supernatant, containing swim-up spermatozoa, was aspirated and the number of motile spermatozoa was evaluated using a Neubaur haemocytometer.

Generation of ROS

ROS were generated using the xanthine–xanthine oxidase (X/XO) system described by McCord and Fridovich (1968). Stock solutions of xanthine oxidase (Sigma, St Louis, MO, USA) (50 mU) and xanthine (Sigma) (200 μM) were prepared in HTF and added to 30 samples immediately after swim-up. H_2O_2, ${}^{\bullet}O_2{}^-$, and OH- were the free radicals known to be generated using this system. At timed intervals of 15 min, 30 min, 1 h and 2 h of ROS exposure, 20 μl of the sample was removed and fixed with 1% formaldehyde on a slide and allowed to air dry.

The concentration of ROS produced by this system was assessed by a chemiluminescence assay using luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) (Sigma). For analysis, 20 μl of luminol solution (5 mM in water) was added to each sperm aliquot. Chemiluminescence after adding luminol was measured using a Berthold luminometer (Lumat LB 9501, Wallace Inc., Gaithersburg, MD, USA). ROS production was expressed as photons $\times 10^5$ (c.p.m.) counted in 20 s. One aliquot was used to measure the background luminescence for each specimen before adding luminol. The background reading was subtracted from the actual test value to obtain the ROS level.

Antioxidants

The second part of this study involved the pretreatment of swim-up samples with antioxidants. Seventeen specimens were divided into five aliquots. The sperm concentration was adjusted in each aliquot with HTF solution so that a concentration of 2×10^6/ml was present in each sample. Antioxidants [N-acetylcysteine (0.1 mM), catalase (500 U/ml), reduced glutathione (10 mM) and hypotaurine (10 mM)] were freshly prepared in HTF and added to the swim-up sample 10 min before X/XO addition. Seven additional sperm-wash samples were pretreated with superoxide dismutase [SOD (100 U/ml)] or a combination of SOD and penicillamine (10 mM), 10 min before X/XO exposure. The same procedure was repeated as above to prepare all antioxidant-pretreated samples for the TUNEL assay.

TUNEL assay

DNA fragmentation in the spermatozoa recovered after exposure to ROS and/or antioxidants, was measured using a modification of the method of terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin end-labelling (TUNEL) which we previously described (Jurisicova et al., 1996; Sun et al., 1997). The air-dried slides were washed in phosphate-buffered saline (PBS), pH 7.4, then spermatozoa were permeabilized with Triton X-100 (Caledon Laboratories Ltd, Georgetown, ON, Canada). Buffer containing 10 U of TdT enzyme (Pharmacia LKB Biotech, Piscataway, NJ, USA) was added and the slides were allowed to incubate at 37°C for 60 min. Following TdT exposure, spermatozoa were treated with a staining buffer containing 1% streptavidin/Texas Red anti-biotin (Calbiochem-Novabiochem Corporation, La Jolla, CA, USA) and incubated at 4°C in the dark for 30 min. The stained cells were washed in PBS and counterstained with 4,6-diamidino-2-phenylindole (DAPI) which stains all chromatin, before analysis.

An ~20 μl of spermatozoa from each swim-up sample was removed and fixed before xanthine oxidase or antioxidants were administered, as control slides for comparison with the treated slides.

Analysis

Using a fluorescence microscope, spermatozoa stained with DAPI were counted manually under UV light. This number represented the total spermatozoa in the field. Red fluorescence labelling was detected using a 640 nm filter on the same field. The number of spermatozoa demonstrating red fluorescence was expressed as a percentage of the total spermatozoa in the field to give an average (± SEM) DNA
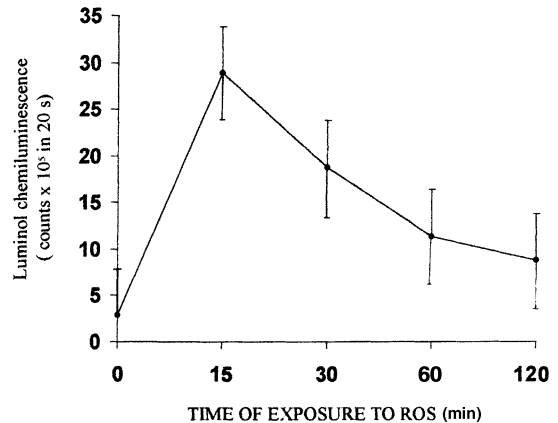

Figure 1. Kinetics of reactive oxygen species (ROS) generation in the xanthine oxidase system used in this study by luminol chemiluminescence.

fragmentation rate. This assessment was performed on 10 fields per sample.

Statistical evaluation

Since the data were not normally distributed, the unpaired Wilcoxon signed-rank test was used for comparison of the DNA fragmentation rate before and at 2 h after ROS exposure. A two-way analysis of variance was used to detect a significant difference between antioxidant treatment groups and the sample group with no antioxidant treatment. A P value of < 0.05 was considered significant.

Results

A total of 54 sperm samples was obtained from males undergoing routine semen analysis. ROS were generated exogenously in these samples and the spermatozoa analysed for DNA fragmentation using the TUNEL method as described above. The mean basal level of ROS in the samples was 2.85×10^5 c.p.m., which represented the amount of free radicals present in the swim-up sample before treatment. ROS were then generated in 30 samples, creating an average ROS concentration of 28.8×10^5 c.p.m. at 15 min which decreased over time (Figure 1). Before treatment, the percentage of spermatozoa with fragmented DNA was <4% in the majority of samples (mean ± SEM was 3.89 ± 2.1), ranging from 0% to 16%. A 4-fold increase in the DNA fragmentation rate was observed after spermatozoa were exposed to ROS for up to 2 h (Figure 2; P = 0.0001). Seven of the spermatozoa-wash samples were pretreated with SOD, or a combination of SOD and penicillamine, 10 min before the addition of X/XO. There was no protection of the spermatozoa against DNA fragmentation by SOD with or without penicillamine (data not shown). The remaining 17 samples were divided into five aliquots each containing $\sim2\times10^6$ spermatozoa/ml. Each sample was pretreated with one of four antioxidants or a combination of these prior to X/XO generation of ROS (Figure 2). The addition of N-acetylcysteine (P = 0.04), hypotaurine (P = 0.01) and reduced glutathione (P = 0.01) was observed to have a significant protective effect on DNA fragmentation. Catalase was ineffective (P > 0.05). The most effective prevention of DNA fragmentation appeared to be the addition
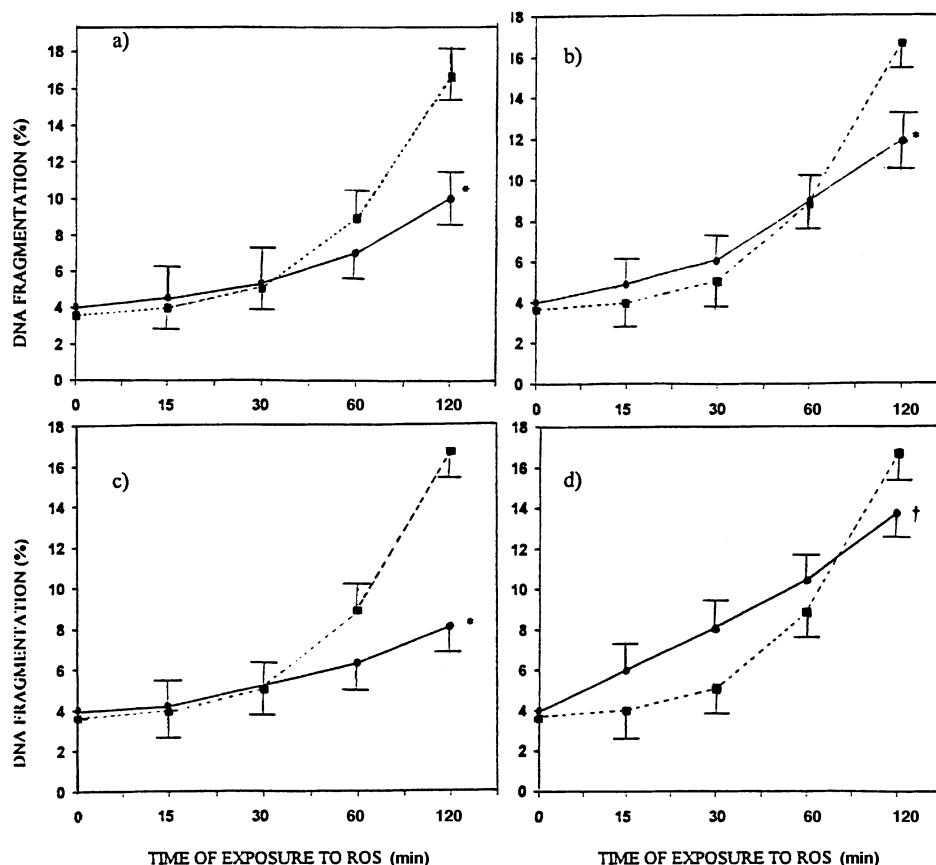
Figure 2. Effect of antioxidant pretreatment on DNA fragmentation in human spermatozoa. (**a**) Reduced glutathione (10 mM);
(**b**) *N*-acetylcysteine (0.1 mM); (**c**) reduced glutathione (10 mM) + hypotaurine (10 mM); (**d**) catalase (500 U/ml). Dotted lines and
■ = control; solid lines and ● = treated. *A significant difference between the antioxidant-treated group and the non-treated control group
was evident, *P* < 0.05. Error bars indicate the SEM; *n* = 17 per treatment. †A significant difference between the antioxidant-treated group
and the non-treated control group was not evident, *P* > 0.05. Error bars indicate the SEM; *n* = 17. ROS = reactive oxygen species.

of the combination of two antioxidants, reduced glutathione and hypotaurine (*P* = 0.0001) (Figure 2).

Discussion

Depending on the nature and concentration of the particular ROS involved, ROS can have beneficial or detrimental effects on sperm function (de Lamirande and Gagnon, 1995). Free radicals are necessary for maintaining hyperactivation and the ability of spermatozoa to undergo the acrosome reaction (de Lamirande and Gagnon, 1993; de Lamirande *et al.*, 1993). Under normal physiological conditions, seminal plasma contains low levels of ROS, contributed by both leukocytes and the spermatozoa themselves (Aitken *et al.*, 1992). In the semen of oligozoospermic men, the predominant source of ROS is spermatozoa (Aitken *et al.*, 1992). As many as 25% of semen samples from infertile men have been demonstrated to have increased levels of ROS (Iwasaki and Gagnon, 1992), suggesting that spermatozoa with impaired function may produce excessive ROS. Free radicals have been hypothesized to play a causative role in the aetiology of defective spermatozoa function through peroxidation of the unsaturated fatty acids within the sperm plasma membrane (Aitken *et al.*, 1993). High levels of ROS were correlated with decreased concentrations of motile spermatozoa with poor sperm motility.

In the present study, we demonstrate that free radicals, such as $^{\bullet}O_2^-$, H_2O_2 and OH^- created by the X/XO system, can cause DNA damage in human spermatozoa when exposed for time periods consistent with clinical sperm preparation techniques for ICSI or IVF. In comparison with other cells, sperm nuclear chromatin is highly condensed, primarily due to the replacement of histones by protamine, with increased disulphide bond formation (Balhorn, 1982). Therefore, sperm DNA is normally highly resistant to physical or chemical denaturation. However, in subfertile men, defects in chromatin condensation have been shown to result in increased DNA instability and sensitivity to denaturing stress (Balhorn *et al.*, 1988; Manicardi *et al.*, 1995). We have confirmed this finding recently by demonstrating that spermatozoa of poor quality used for ICSI contained higher amounts of fragmented DNA than better quality spermatozoa from men in the IVF programme, or from normal fertile males (Lopes *et al.*, 1998). From the results of the previous studies described above, we speculate that spermatozoa from poor-quality samples not only have the capability to produce high levels of ROS, but also are much more susceptible to DNA damage caused by oxidative stress.

The present study utilized the specific activity of the enzyme terminal transferase (TdT) to incorporate polymers of biotinylated deoxyuridine onto the 3′-OH ends of DNA. The

signal was amplified by streptavidin/Texas Red conjugate. Spermatozoa with normal DNA, in which the 3′-OH ends of DNA are capped by telomeres, demonstrated no fluorescence while those with fragmented DNA (multiple chromatin 3′-OH ends) fluoresced brightly. We have previously used the same technique, along with DAPI staining of chromatin, to confirm the occurrence of DNA fragmentation associated with apoptosis in fragmented human embryos (Jurisicova *et al.*, 1996) and to demonstrate sperm DNA fragmentation in washed semen samples used for IVF (Sun *et al.*, 1997) and ICSI (Lopes *et al.*, 1998).

It has been reported that a key factor in the production of ROS by damaged or deficient spermatozoa may be the preparation technique. Sperm washing and swim-up is associated with repeated centrifugation which may lead to significantly higher ROS production (Agarwal *et al.*, 1994). This finding is of particular relevance during preparation of spermatozoa for ICSI where poor-quality spermatozoa are characteristic of samples selected for this procedure. The sperm washing procedures could trigger increased production of ROS which begin to accumulate in the sample; thus, prolonged exposure of spermatozoa to ROS occurs after swim-up is complete while oocytes are prepared for injection. Our results indicate that spermatozoa exposed to ROS for >1 h have an increased DNA fragmentation rate.

To counteract the damaging effects of ROS, a variety of antioxidants are present within the seminal plasma and in spermatozoa (Alvarez and Storey, 1989; Lewis *et al.*, 1997). Once seminal plasma is washed away during sperm preparation for assisted reproduction techniques, much of the antioxidant protection is lost, since the antioxidant content of human spermatozoa is limited (Alvarez and Storey, 1989; Lewis *et al.*, 1997). Cellular damage arises when the equilibrium between the amount of ROS produced and that scavenged by antioxidants is disturbed, and this imbalance has been shown to correlate with idiopathic infertility (Sharma and Agarwal, 1996).

Our results demonstrate that a combination of reduced glutathione and hypotaurine, which are considered to be suicide antioxidants, was most protective against DNA fragmentation. In addition to having the ability to neutralize $^{\bullet}O_2^-$, reduced glutathione is also a substrate for glutathione peroxidase which metabolizes H_2O_2 and OH^-. Hypotaurine on the other hand, is able to react directly with cytotoxic aldehydes produced during lipid peroxidation and thus protects the thiol groups on the sperm plasma membrane. Baker *et al.* (1996) have also demonstrated the effectiveness of glutathione in combination with hypotaurine on spermatozoa motility. Catalase, which scavenges H_2O_2, was ineffective in preventing sperm DNA damage, suggesting that although H_2O_2 may be an important initiator of lipid peroxidation, it has little effect on chromatin.

In summary, we have demonstrated that exogenous ROS generation causes an increase in DNA fragmentation in human spermatozoa after swim-up. An increase in oxidative damage to the sperm membrane, intracellular proteins and DNA is associated with alterations in signal transduction mechanisms that can affect fertility (Sikka *et al.*, 1995). We also showed that the administration of antioxidants prevented the amount of DNA damage observed. Poor quality spermatozoa such as samples selected for ICSI, are capable of producing increased levels of ROS during routine sperm preparation and our results indicate that spermatozoa are prone to DNA damage when exposed to ROS for at least 1 h. DNA damage in turn could lead to the failure of fertilization if spermatozoa containing fragmented DNA are selected for injection (Lopes *et al.*, 1998). Previous studies have used antioxidants to improve sperm function (Krausz *et al.*, 1994; Lenzi *et al.*, 1994) and our results suggest an additional therapeutic reason for supplementation of media with free radical scavengers. Further studies are required to determine the amount of endogenous ROS produced by spermatozoa during such procedures, and to develop the optimal combination of antioxidants to supplement sperm preparation media to protect against DNA fragmentation.

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