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Relative Impact of Oxidative Stress on the Functional Competence and Genomic Integrity of Human Spermatozoa[1]

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ABSTRACT

Reactive oxygen metabolites are known to disrupt sperm-oocyte fusion, sperm movement, and DNA integrity; however, the relative sensitivities of these elements to oxidative stress are unknown. In this study these factors were assessed in human spermatozoa exposed to increasing levels of oxidative stress achieved through the stimulation of endogenous oxidant generation with NADPH or direct exposure to hydrogen peroxide. At low levels of oxidative stress, DNA fragmentation was significantly reduced while the rates of sperm-oocyte fusion were significantly enhanced. As the level of oxidative stress increased, the spermatozoa exhibited significantly elevated levels of DNA damage ($p < 0.001$) and yet continued to express an enhanced capacity for sperm-oocyte fusion. At the highest levels of oxidative stress, extremely high rates of DNA fragmentation were observed but the spermatozoa exhibited a parallel loss in their capacities for movement and oocyte fusion. These studies emphasize how redox mechanisms can either enhance or disrupt the functional and genomic integrity of human spermatozoa depending on the intensity of the oxidative stimulus. Because these qualities are affected at different rates, spermatozoa exhibiting significant DNA damage are still capable of fertilizing the oocyte. These results may have long-term implications for the safety of assisted conception procedures in cases associated with oxidative stress.

INTRODUCTION

The potential capacity of oxidative stress to disrupt the functional competence of human spermatozoa was first recognized in 1943 by MacLeod [1] when he recorded the negative impact of high oxygen tensions on sperm motility. Furthermore, the significance of reactive oxygen species (ROS), and specifically hydrogen peroxide (H_2O_2), in the induction of this motility loss was indicated by the ameliorating action of catalase [1]. Subsequent studies have generated definitive evidence for the generation of ROS by human spermatozoa [2–4] and demonstrated a significant increase in this activity in cases of male infertility [5–7]. This enhanced free radical-generating activity appears to be associated with the retention of excess residual cytoplasm by the spermatozoa during the final stages of spermiogenesis [8, 9]. The additional intracellular space resulting from this developmental defect results in the presence of an excessively high cellular content of cytoplasmic enzymes such as creatine kinase, lactic acid dehydrogenase, and glucose-6-phosphate dehydrogenase (G6PDH) in affected cells [8–10]. The high intracellular levels of G6PDH are thought to stimulate the generation of NADPH through the hexose monophosphate shunt, at levels that exceed the demands of the glutathione cycle [9]. The excess NADPH generated as a consequence of this enhanced hexose monophosphate shunt activity is then held to stimulate the generation of superphysiological levels of ROS by fueling the activity of a putative NADPH oxidase located in the sperm plasma membrane [9, 11]. This oxidase system plays an important biological role in supporting the tyrosine phosphorylation events associated with sperm capacitation [12–14] as a result of which spermatozoa gain the competence to respond to the calcium transients generated by physiological agonists such as progesterone and zona pellucida glycoprotein 3 [13]. However, when produced in excess, ROS, particularly H_2O_2 [15–17], generated by the spermatozoon's oxidase system attack the high concentrations of polyunsaturated fatty acid in the sperm plasma membrane, initiating a lipid peroxidation cascade [18, 19]. Such peroxidative damage to the sperm plasma membrane leads to a loss of membrane fluidity and integrity as a result of which the spermatozoa lose their competence to participate in the membrane fusion events associated with fertilization [19–21]. In addition to the cytotoxic effects of ROS generated from intracellular sources, spermatozoa may also be damaged by toxic oxygen metabolites generated by leukocytes infiltrating the ejaculate. Such damage is particularly significant in the context of assisted conception therapy, where leukocyte contamination of washed sperm preparations is thought to be a major factor determining the success of fertilization in vitro [22, 23].

In addition to peroxidative damage to the sperm plasma membrane, ROS are also known to attack DNA, inducing strand breaks and oxidative base damage in human spermatozoa [24, 25]. Despite the prevalence of oxidative DNA damage in human spermatozoa, it is not known whether such damaged cells are capable of fertilization. Existing data on the relationship between DNA damage and semen quality are confusing. Thus, Hughes et al. [24] found no differences in baseline DNA damage in the spermatozoa of a cohort of asthenozoospermic patients compared with a group of normozoospermic donors. In contrast, Sun et al. [26] observed negative correlations between DNA fragmentation and semen quality as reflected by sperm motility, morphology, and count. Furthermore, Aravindan et al. [27] found a strong correlation between DNA strand breaks and the susceptibility of spermatozoa to low pH-induced DNA denaturation, which is, in turn, negatively correlated with the fertilizing potential of the spermatozoa [28].

When a spermatozoon is subjected to oxidative stress, it is clear that both the plasma membrane and the integrity of the DNA will come under attack. Whether a spermatozoon with oxidatively damaged DNA is still competent to fertilize an oocyte will clearly depend on the relative rates at which DNA integrity and the individual components of sperm function are lost following an oxidative stress. To

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date we know that sperm motility [15, 16], the competence to undergo the acrosome reaction [17], the ability to fuse with the vitelline membrane of the oocyte [15, 29], and DNA integrity [24, 25, 30] are all susceptible to oxidative damage. However, we do not know the order in which these structures and functions are lost following an oxidative insult and thus the possibility that spermatozoa with oxidatively damaged DNA might generate an embryo. The present study was designed to address this question.

MATERIALS AND METHODS

Sperm Preparation

This study was based upon semen samples donated by a panel of healthy normozoospermic donors after 2 or 3 days abstinence. All samples were produced into sterile containers and left for at least 30 min to liquefy before processing.

The spermatozoa were isolated by discontinuous Percoll gradient centrifugation using a simple 2-step design incorporating 44% and 88% Percoll. Isotonic Percoll was created by supplementing 20 ml of 10-strength concentrated Earle's medium (Gibco, Paisley, UK) with 3 ml of 20% albuminar (Armour Pharmaceutical Company, Eastbourne, UK), a pasteurized human serum albumin preparation in an aqueous base stabilized with 16 mM sodium caprylate and 16 mM sodium acetyltryptophanate, 6 mg sodium pyruvate, 0.74 ml of a 60% sodium lactate syrup, and 2 ml of penicillin/streptomycin (10 000 IU/ml penicillin and 1000 μg/ml streptomycin; Gibco) and adding 180 ml of Percoll (Pharmacia, Uppsala, Sweden). The medium used to dilute the isotonic Percoll was Biggers, Whitten and Whittingham medium (BWW) supplemented with 20 mM Hepes and 0.3% albuminar [31]. Semen (1–3 ml) was layered on the top of each gradient and centrifuged at 500 × *g* for 20 min. Purified populations of highly motile spermatozoa were subsequently recovered from the base of the 88% Percoll fraction, washed with 7 ml BWW, centrifuged at 500 × *g* for 5 min, and resuspended at a concentration of 2×10^7/ml.

For these experiments the spermatozoa were incubated at a final concentration of 1×10^7/ml for 2.5 h in the presence of oxidant (12.5–200 μM H_2O_2 or 1.25–20 mM NADPH) and 3.0 mM pentoxifylline to promote capacitation. At the end of this capacitation period, 5 μM progesterone was added to the sperm suspensions, and the incubations were continued for a further 30 min before being pelleted at 500 × *g* for 5 min and resuspended in fresh medium BWW. At this point, aliquots of the sperm suspension were removed for computer-aided semen analysis (CASA), determination of DNA damage using a comet assay, and evaluation of sperm function.

Sperm-Oocyte Fusion

In order to monitor the ability of human spermatozoa to exhibit a functional acrosome reaction accompanied by the concomitant generation of a fusogenic equatorial segment, zona-free hamster ova were used in a heterologous in vitro fertilization assay [32, 33]. For this assay, the progesterone-treated spermatozoa were resuspended at a concentration of 1×10^7/ml and dispersed into 50-μl droplets under liquid paraffin. Zona-free hamster oocytes were prepared as described previously [33] and dispersed into the droplets at 5 oocytes per drop and at least 15 oocytes per sample. Each experiment was conducted on at least 3 independent semen samples, so that at least 45 oocytes were used in the assessment of each treatment. After a further 3-h incubation at 37°C, the oocytes were recovered from the droplets, washed free of loosely adherent spermatozoa, compressed to a depth of about 30 μm under a 22 × 22-mm coverslip on a glass slide, and assessed for the presence of decondensing sperm heads with an attached or closely associated tail by phase-contrast microscopy. The number of spermatozoa penetrating each egg was assessed and expressed in terms of the degree of polyspermy (total number of penetrations per total number of oocytes) observed [33].

CASA

Sperm movement characteristics were assessed using the Hamilton-Thorn Motility Analyzer (HTMA-IVOS version 10.5; Hamilton Thorne Research Inc., Beverly, MA). In outline, this unit comprised an internal microscope system with a heated stage (37°C) and stroboscopic light source that illuminated the specimen with a series of phase-locked light flashes, acquiring frames at 25 Hz. The images were fed into an integrated IBM-compatible computer that analyzed the data according to a series of preprogrammed algorithms.

The HTMA-IVOS settings employed in the study were minimum contrast, 10; minimum cell size, 4 pixels; low and high size gates, 0.4 and 1.6, respectively; low and high intensity gates, 0.5 and 2.0, respectively; nonmotile head size, 12 pixels; nonmotile cell intensity, 130; slow cells immotile; and magnification factor, 2.33. Thirty frames (1.2 sec) were collected for analysis.

The samples were loaded onto a 20-μm-deep Microcell chamber (Conception Technologies, La Jolla, CA), which had been preheated to 37°C, and analyzed. Accurate identification of nonmotile sperm cells versus debris and round cells was achieved by replaying the stored video frames and adjusting the static intensity, elongation, and size limits appropriately. Data from at least 200 motile spermatozoa were collected for each specimen analyzed.

The motility parameters assessed included curvilinear velocity (VCL, μm/sec), straight line velocity (VSL, μm/sec), average path velocity (VAP, μm/sec), amplitude of lateral head displacement (ALH, μm), straightness (STR = VSL/VAP × 100, %), % motile cells, % rapid VAP (> 25 μm/sec), % medium VAP (10–25 μm/sec), and % progressive cells (STR > 75%). In addition, the DANCEMEAN (ALH/linearity × 100, μm) was calculated and used to quantify the percentage of hyperactivated (HA) and transitional phase (TP) cells according to the criteria laid down by Sukcharoen et al. [34].

Assessment of DNA Damage

The alkaline single-cell gel electrophoresis (comet) assay was based on existing methods [24, 35, 36]. Unless otherwise stated, molecular grade, DNase-free reagents (Sigma, Poole, UK) were used throughout. Microscope slides (Select; Chance Propper Ltd., Smethwick, UK) were used for the assay, and each slide was prepared in the following manner. The slide was dipped in a solution of 1% multipurpose agarose (Boehringer Mannheim, Lewes, UK) dissolved in 0.01 M PBS and air dried overnight at room temperature. A solution (160 μl) of 0.6% multipurpose agarose, dissolved in PBS, was then placed on the slide, covered with 24 × 50-mm coverslip (Chance Propper Ltd.), and left to solidify at room temperature. Ten microliters of spermatozoa at 6.6×10^6 ml in BWW was then mixed with 75

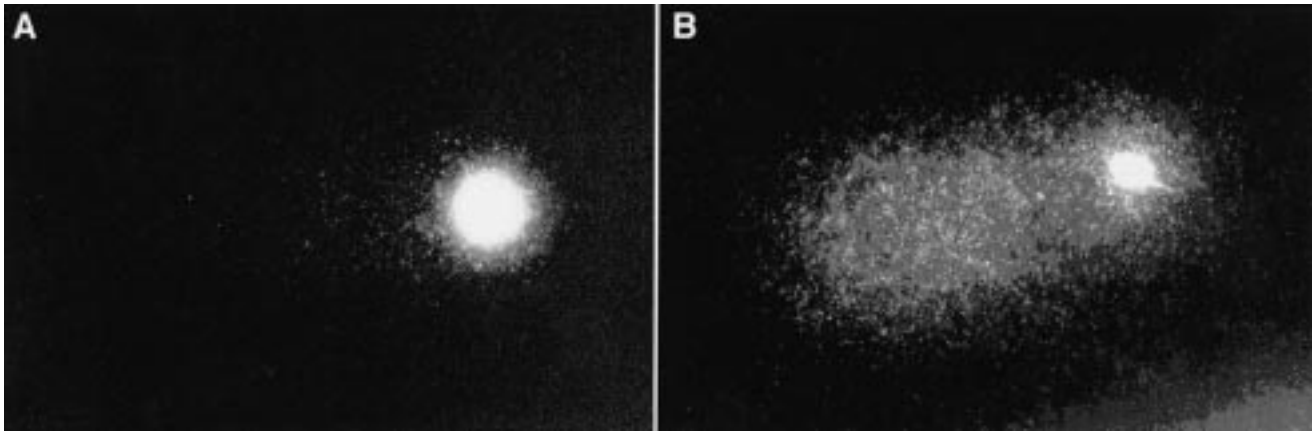

FIG. 1. An illustration of the outcome of the single-cell electrophoresis comet assay for DNA fragmentation. **A** depicts a control spermatozoon in which a majority of the DNA is seen to migrate as a single cohesive mass. **B** illustrates the change in migratory behavior of sperm DNA after the induction of oxidative damage with 100 μM H_2O_2. Under these circumstances a comet-like structure is created, with the fragmented DNA forming the "tail." The percentage of the ethidium bromide-dependent fluorescent signal present in the comet's tail provides a measure of DNA fragmentation in individual cells.

μl of a 0.5% low-melt agarose solution (Amresco, Solon, OH) dissolved in PBS. This mixture was then added to the slide, overlaid with a coverslip, and allowed to solidify on a prechilled (4°C) tray for several minutes. A final layer of a 0.5% low-melt agarose was added to the slide and allowed to solidify at 4°C for at least 1 h.

After removal of the coverslip, the slides were immersed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl [Sigma], 10% dimethylsulfoxide [DMSO], and 1% Triton X-100, pH 10) for 1 h at 4°C. The lysis solution was drained from the slides and replaced with a solution of proteinase K (Amresco; 100 μg/ml in 2.5 M NaCl, 100 mM EDTA, 10% DMSO, pH 7.4) and incubated overnight at 37°C. After the proteinase K solution was drained, the slides were immersed in a horizontal gel tank filled with alkaline buffer (300 mM sodium hydroxide, 1 mM EDTA, pH 12.3) for 20 min to allow the DNA to unwind. The buffer level was adjusted to a covering height of approximately 0.25 cm above the slides, and electrophoresis was carried out for 4 min at 25 V (0.862 V/cm). Slides were then placed in Coplin jars filled with 0.4 M Tris-HCl (pH 7.4) for 5 min and washed three times using fresh buffer. After rinsing, the slides were drained and immersed in Coplin jars containing 100% ethanol for 5 min in order to precipitate the DNA and dehydrate the agarose. The slides were then air dried overnight and stored in a box prior to scoring. Ethidium bromide was used to stain the sperm DNA (50 μl at 20 μg/ml dissolved in distilled water), and imaging was carried out on a Leitz (Leitz Wetzlar GBH, Wetzlar, Germany) fluorescent microscope (excitation filter 515–560 nm, dichroic 580 nm, suppression filter 580 nm) connected to a charge-coupled device camera. For each sample, 4 replicate slides were prepared, and 100 randomly selected cells were scored on each slide. The percentage of tail DNA was evaluated using the Komet version 1.0 image analysis system (Kinetic Imaging, Liverpool, UK) running on an IBM-compatible personal computer (Windows 3.1) and downloaded into an Excel (Microsoft, Redmond, WA) spreadsheet for analysis. An illustration of the outcome of this particular assay for DNA damage is presented in Figure 1.

Statistics

The data were examined by ANOVA for repeated measures by using the Statview program (Abacus Concepts, Berkeley, CA) on an Apple (Cupertino, CA) Macintosh Centris 650 computer. Differences between individual groups were examined with Fisher's protected least-significant-difference test. Stepwise multiple regression was used to identify groups of independent variables that would predict the dependent variable (DNA damage) with optimal efficiency. The relationship between the observed levels of DNA damage and those predicted on the basis of the multiple regression equation was then plotted to illustrate the overall accuracy of the latter. All data are presented as the mean value ± SE, and every experiment was conducted on at least 3 independent samples from different donors.

RESULTS

Induction of ROS with NADPH

Addition of NADPH to purified suspensions of human spermatozoa has been shown to result in the dose-dependent induction of ROS [11]. Since this co-enzyme is highly membrane impermeant, millimolar concentrations of NADPH have to be added to the medium bathing the spermatozoa in order to raise the intracellular concentration of this nucleotide to the point where significant ROS induction occurs [11]. Using this strategy, suspensions of human spermatozoa were exposed to increasing levels of endogenous ROS generation for 3 h prior to an analysis of their functional and genomic integrity.

The induction of oxidative stress was associated with highly significant changes ($p < 0.001$) in the level of DNA fragmentation recorded in the sperm nuclei with the comet assay (Fig. 2A). At the lowest level of induced ROS generation (1.25 mM NADPH), there was a significant decline in the levels of DNA damage ($p < 0.001$). However, above this threshold, the stimulation of endogenous ROS production was associated with a dose-dependent increase in DNA strand breakage, such that at the highest dose of NADPH examined, around 75% of the DNA in the sperm nuclei was fragmented (Fig. 2A).

Analysis of the competence of the spermatozoa for sperm-oocyte fusion also revealed highly significant changes ($p < 0.001$) in response to the induction of endogenous ROS generation with NADPH. At the lowest levels of ROS generation (1.25 mM), the spermatozoa exhibited a significant increase in sperm-oocyte fusion rates (Fig.

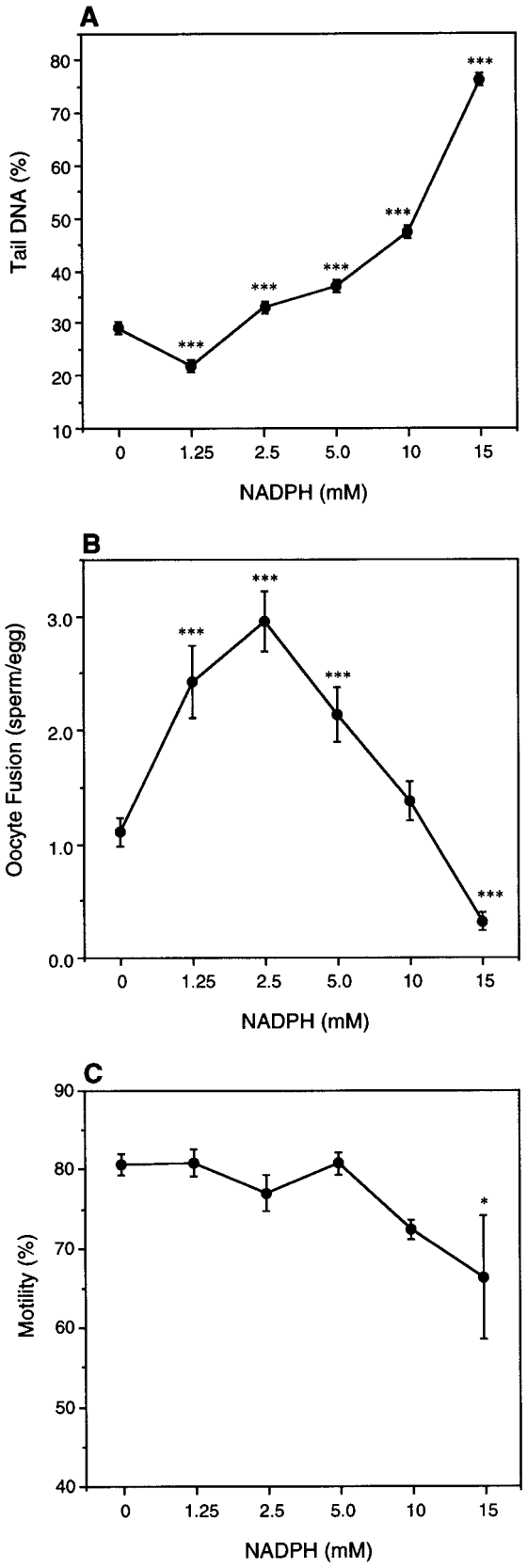

FIG. 2. Analysis of the impact of endogenous ROS generation on the functional and genomic integrity of human spermatozoa. Endogenous ROS generation was stimulated with NADPH [11]. **A**) DNA damage according to the comet assay; **B**) sperm-oocyte fusion in response to 5 μM progesterone; **C**) percentage of motile spermatozoa. ***$p < 0.001$; *$p < 0.05$ for 5 independent donor samples. A square-root transformation was used to ensure even distribution of data points along the x-axis.

2B) in concert with the decrease in DNA damage (Fig. 2A). When the level of ROS generation was raised still further by addition of 2.5 and 5.0 mM NADPH, sperm-oocyte fusion rates remained significantly elevated above control levels ($p < 0.001$), even though DNA damage in these populations of spermatozoa was significantly elevated (Fig. 2, A and B). It was only at the highest levels of induced ROS production that the levels of sperm-oocyte fusion declined below control values ($p < 0.001$; Fig. 2B).

In these same cell populations, the percentage of motile spermatozoa remained unchanged across the range of NADPH doses except at the highest level of oxidative stress examined (Fig. 2C). At this point, when the sperm DNA was highly fragmented and sperm-oocyte fusion levels were diminished, the spermatozoa showed a slight and highly variable decline ($p < 0.05$) in percentage motility (Fig. 2C). Despite the general preservation of percentage motility in the face of oxidative stress, analysis of the detailed movement characteristics of the spermatozoa by CASA revealed highly significant changes ($p < 0.001$) in response to the induction of endogenous ROS generation. The most sensitive attributes of sperm movement were the continuous velocity measurements (VAP, VCL, VSL) and ALH, all of which revealed a moderate but statistically significant ($p < 0.001$) decline in velocity at the lowest levels of NADPH stimulation (1.25–5 mM; Fig. 3, A–D). However, this plateau of suppression was followed by a sudden collapse of sperm movement at the two highest levels of endogenous ROS generation investigated (Fig. 3, A–D). Percentage-based parameters such as % STR or % rapid were less responsive to low levels of induced ROS production (1.25–5.0 mM NADPH) but showed the same dramatic decline at the two highest doses of oxidative stress examined (Fig. 3, E and F). Measurements of transitional and hyperactivated motility (Fig. 4A) revealed extremely low levels of hyperactivated movement under the conditions employed in this study, notwithstanding the fact that the spermatozoa were capacitated, judging from the levels of sperm-oocyte fusion observed in response to progesterone [37]. High levels of transitional phase motility were observed, however, which were sustained under conditions of oxidative stress except at the two highest doses of NADPH, when a significant suppression of this form of movement was observed (Fig. 4A).

A linear regression analysis in which DNA fragmentation was used as the dependent variable emphasized that the induction of DNA damage by oxidative stress exhibited a significant negative correlation with every other aspect of sperm function examined, including sperm-oocyte fusion ($r = -0.567$; $p < 0.001$), and particularly the CASA variables, VCL ($r = -0.675$, $p < 0.001$), VSL ($r = -0.737$; $p < 0.001$), VAP ($r = -0.730$, $p < 0.001$), STR ($r = -0.650$, $p < 0.001$), progressive motility ($r = -0.623$, $p < 0.001$), and ALH ($r = -0.601$, $p < 0.001$). Stepwise multiple regression analysis revealed that DNA damage to the spermatozoa could be predicted very efficiently ($R = 0.879$) on the basis of three variables (hyperactivation, progressive motility, and VSL), of which VSL was the first incorporated and most significant variable (Fig. 5).

Exposure to H_2O_2

In order to confirm that the changes observed on exposure of spermatozoa to NADPH were due to ROS generation, the experiments described above were repeated using H_2O_2 to produce the oxidative stress.

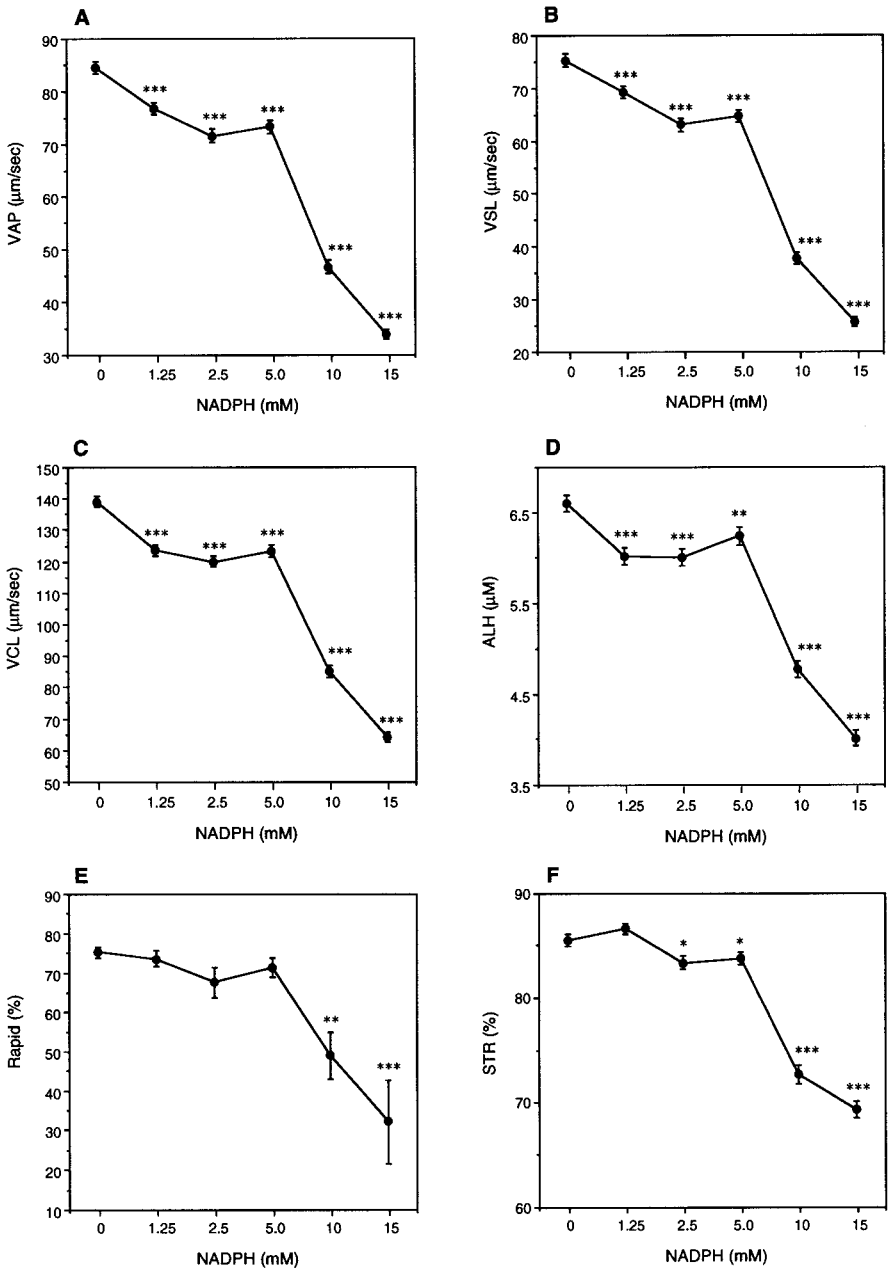

FIG. 3. Analysis of the quality of sperm movement in the face of elevated levels of endogenous ROS generation stimulated with NADPH [11]. The movement characteristics illustrated are **A**) VAP, **B**) VSL, **C**) VCL, **D**) ALH, **E**) percentage rapid, **F**) STR. $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$ for 5 independent donor samples. A square-root transformation was used to ensure the even distribution of data points along the x-axis.

At the lowest dose of H_2O_2 examined (12.5 μM), a highly significant decline ($p < 0.001$) in the levels of DNA damage recorded in the spermatozoa was observed (Fig. 6A). As the level of oxidative stress increased over the range of 25–100 μM H_2O_2, a significant increase in DNA damage over control values was detected ($p < 0.001$); this rose dramatically at the highest dose examined (200 μM H_2O_2), at which point approximately 90% of the DNA in the sperm heads was fragmented.

Analysis of the ability of the spermatozoa to fuse with the oocyte after exposure to H_2O_2 revealed a highly significant increase in the rates of sperm-oocyte fusion at the two lowest levels of oxidative stress (12.5 and 50 μM) examined, followed by a dramatic decline over the range 50–200 μM H_2O_2 (Fig. 6B).

H_2O_2 had a much less dramatic effect on sperm motility, since the percentage of motile cells was sustained at a high level across the entire H_2O_2 dose range with the exception of the highest dose (200 μM), at which point a slight, and highly variable, suppression of motility was noted (Fig. 6C). Analysis of the quality of sperm movement by CASA revealed little change at the lowest concentration of H_2O_2 assessed (12.5 μM) with the exception of a significant increase in STR and slight declines in ALH and VCL ($p < 0.001$; Fig. 7, C, D, and F). Thereafter the quality of sperm movement gradually declined in line with H_2O_2 concentration for continuous variables such as VAP, VSL, VCL, and ALH (Fig. 7, A–D). STR was more resistant to oxidative stress but declined significantly at the two highest doses of H_2O_2 examined (Fig. 7F), while the percentage of rapidly moving cells did not change significantly until the 200 μM dose, at which point a marked decline was observed (Fig. 7E).

Linear regression analysis using DNA fragmentation as the dependent variable showed significant ($p < 0.001$) negative correlations with every aspect of sperm movement examined, particularly VSL ($r = -0.726$, $p < 0.001$), VAP ($r = -0.703$, $p < 0.001$), VCL ($r = -0.660$, $p < 0.001$),

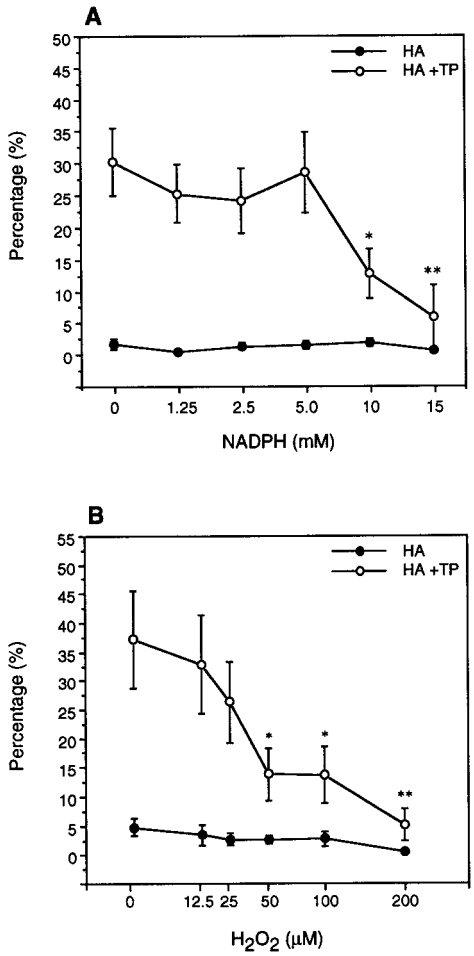

FIG. 4. Analysis of the changes in TP and HA movements in response to oxidative stress. TP and HA movements were defined according to the criteria developed by Sukcharoen et al. [34] for analyses at 25 Hz. **A**) Stimulation of endogenous ROS generation with NADPH [11]. **B**) Exposure to H_2O_2. $^{**}p < 0.01$; $^{*}p < 0.05$ for 5 independent donor samples. A square-root transformation was used to ensure the even distribution of data points along the x-axis.

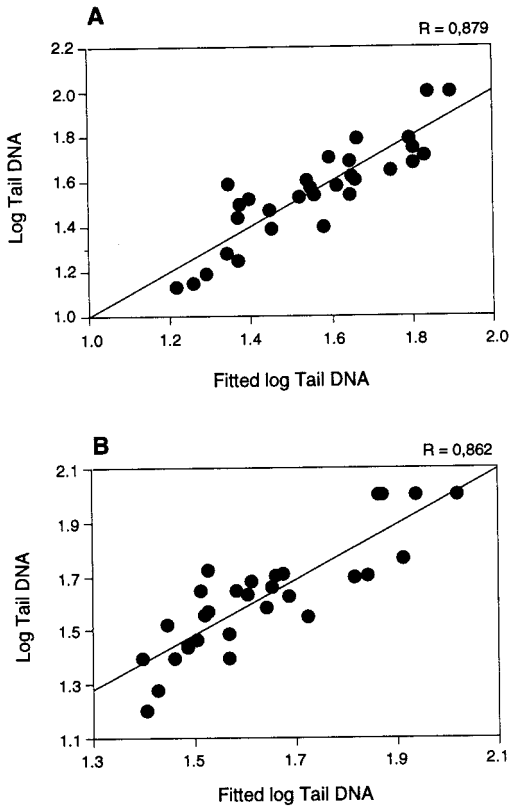

FIG. 5. Stepwise linear regression analysis of the relationship between DNA fragmentation in human sperm populations and the quality of sperm movement. Relationships between actual DNA damage observed in the spermatozoa and predictions of damage based on the quality of sperm movement are presented as scattergram plots. **A**) The NADPH data set, illustrating the relationship between observed DNA damage and that predicted by a multiple regression equation based on VSL, HA, and percentage progressive spermatozoa. **B**) The H_2O_2 data set; prediction of DNA damage based on VSL, HA, and the percentages of spermatozoa that were progressive and exhibiting medium VAP (10–25 μm/sec).

STR ($r = -0.711$, $p < 0.001$), progressive ($r = -0.790$, $p < 0.001$), rapid ($r = -0.741$, $p < 0.001$), and ALH ($r = -0.558$, $p < 0.01$). However, sperm-oocyte fusion did not exhibit a simple linear correlation with DNA damage, because of the stimulatory effects of low-level H_2O_2 exposure on this aspect of sperm function. Stepwise regression analysis revealed that the level of DNA fragmentation could be predicted very accurately (R = 0.862) on the basis of four variables, comprising VSL, a VAP of 10–25 μM/sec, and the percentages of cells that were progressive and hyperactivated.

DISCUSSION

The results obtained in this study emphasize that redox-regulated pathways can play both physiological and pathological roles in the regulation of human sperm function [20]. These experiments also demonstrate that the functional competence of these cells and the integrity of their DNA are affected by ROS at different rates, in different ways. Low levels of oxidative stress, created by exposing spermatozoa to 1.25 mM NADPH or 12.5 μM H_2O_2, induced a stimulation of sperm-oocyte fusion and a reduction in DNA fragmentation. The beneficial effects of low-level ROS exposure on sperm-oocyte fusion presumably reflect the positive role that these molecules are thought to play in the attainment of a capacitated state [12–14, 20, 38–41]. The bioassay employed in these studies utilized the extragenomic action of progesterone to generate a calcium transient in the acrosomal domain of the spermatozoa, precipitating the acrosome reaction and thus sperm-oocyte fusion [37, 42, 43]. The ability of such progesterone-induced calcium transients to activate human spermatozoa is exquisitely dependent on the capacitation status of these cells [37, 38]. Key mediators of sperm capacitation are reactive oxygen metabolites, in the form of both superoxide anion and hydrogen peroxide [39]. Superoxide anion is responsible for the activation of hyperactivated motility [40], while hydrogen peroxide appears to be a significant factor in controlling the tyrosine phosphorylation events associated with sperm capacitation [12, 44, 45]. Tyrosine phosphorylation is an important factor in the attainment of a capacitated state [46] that sensitizes human spermatozoa to the calcium transients generated by progesterone [13]. Since both hydrogen peroxide and NADPH have been shown to induce tyrosine phosphorylation in human spermatozoa [12, 44], the beneficial effects recorded in the present study are entirely consistent with published data supporting the existence of redox-regulated mechanisms for promoting sperm capacitation.

The beneficial effects of low-level oxidative stress on

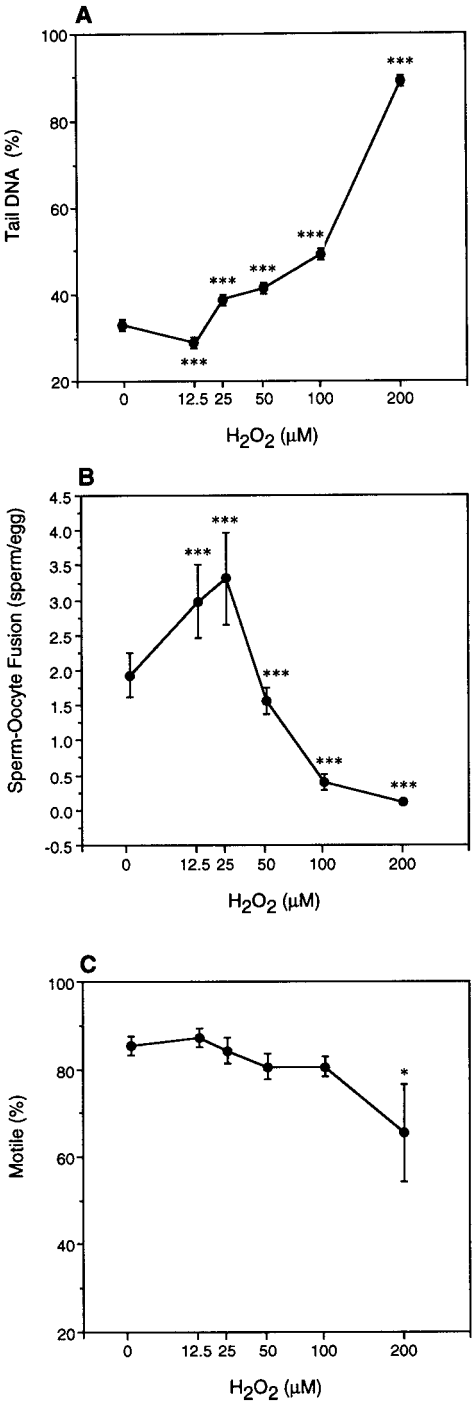

FIG. 6. Analysis of the impact of H_2O_2 on the functional and genomic integrity of human spermatozoa. **A**) DNA damage according to the comet assay; **B**) sperm-oocyte fusion in response to 5 μM progesterone; **C**) percentage of motile spermatozoa. ***$p < 0.001$; *$p < 0.05$ for 5 independent donor samples. A square-root transformation was used to ensure the even distribution of data points along the x-axis.

DNA damage are also consistent with recent studies [47] suggesting a role for ROS in the induction of a process, sperm chromatin compaction, that is thought to be of physiological importance in protecting the sperm genome from oxidative attack [48, 49]. The notion of the possible involvement of reactive oxygen metabolites in mediating the terminal stages of DNA compaction stems from studies indicating that mammalian spermatozoa possess a phospholipid glutathione peroxidase (PHGPx) that shows activity toward intact phospholipid hydroperoxides and fatty acid hydroperoxides as well as hydrogen peroxide itself [47]. This selenium-containing enzyme is tightly bound to the sperm chromatin [47] and exhibits a thiol oxidase activity that can use not just glutathione as substrate but also the thiol groups associated with the protamines of caput epididymal spermatozoa. This thiol oxidase activity is thought to induce the protamine cross-linking that characterizes the terminal stages of DNA compaction [47]. In order for this enzyme to function, it requires an electron acceptor in the form of hydrogen peroxide, fatty acid hydroperoxide, or phospholipid hydroperoxide. We have recently presented evidence indicating that epididymal spermatozoa are capable of generating ROS via a mechanism that exhibits the general properties of an NADPH oxidase [11, 50]. In light of such data we have suggested that one of the functions of this ROS-generating system is to create the peroxide species that will be used by sperm PHGPx to effect the thiol oxidation stage of DNA compaction [51]. Since thiol oxidation is apparently incomplete in a proportion of human spermatozoa at ejaculation [52], it is possible that the low level of oxidative stress created by 1.25 mM NADPH or 12.5 μM H_2O_2 protects the sperm DNA from fragmentation by stimulating the nuclear PHGPx to promote protamine cross linkage and complete the compaction process.

Thus at low levels of oxidative stress, PHGPx is thought to reduce peroxides arriving at the sperm nucleus, thereby oxidizing protamine thiols while protecting the sperm DNA from oxidative attack. This is the physiological situation. As the levels of oxidative stress increase, however, the limited capacity of the spermatozoon's glutathione cycle would be rapidly overwhelmed [53] and DNA damage induced. This was presumably the situation with the intermediate levels of oxidative stress observed in the presence of 25 μM H_2O_2 or 2.5–5 mM NADPH. At this stage of the dose-response curve, the spermatozoa exhibited significantly elevated levels of DNA damage and yet expressed a capacity for sperm-oocyte fusion that was significantly above normal ($p < 0.001$; Figs. 2 and 6). The clinical implications of these findings are most evident in the context of assisted conception procedures involving the disruption or circumvention of the zona barrier via procedures such as zona drilling, partial zona dissection (PZD), or subzonal insemination. These techniques are practiced in cases in which the male partner suffers from motility defects that render the spermatozoa incapable of penetrating the zona pellucida. Unfortunately, asthenozoospermia is frequently associated with oxidative stress [54] and fragmentation of the sperm DNA [28]. In light of the results obtained in the present study it is possible that with procedures such as PZD, spermatozoa suffering from a moderate degree of DNA damage are more likely to fuse with the vitelline membrane of the oocyte than normal cells, because of the beneficial effects of low-level oxidative stress on sperm capacitation.

It is probable that DNA-damaged spermatozoa suffering from moderate levels of oxidative stress are also able to fertilize oocytes when conventional in vitro fertilization procedures are used. Thus, although the quality of sperm movement was significantly affected at moderate levels of oxidative stress in terms of parameters such as VCL, VAP, VSL, and ALH, it should be recognized that the spermatozoa incubated in 25 μM H_2O_2 or 2.5–5 mM NADPH were still extremely motile; percentage motility was unimpaired, and more than 70% of the spermatozoa were exhibiting rapid, progressive movement with VCL values in

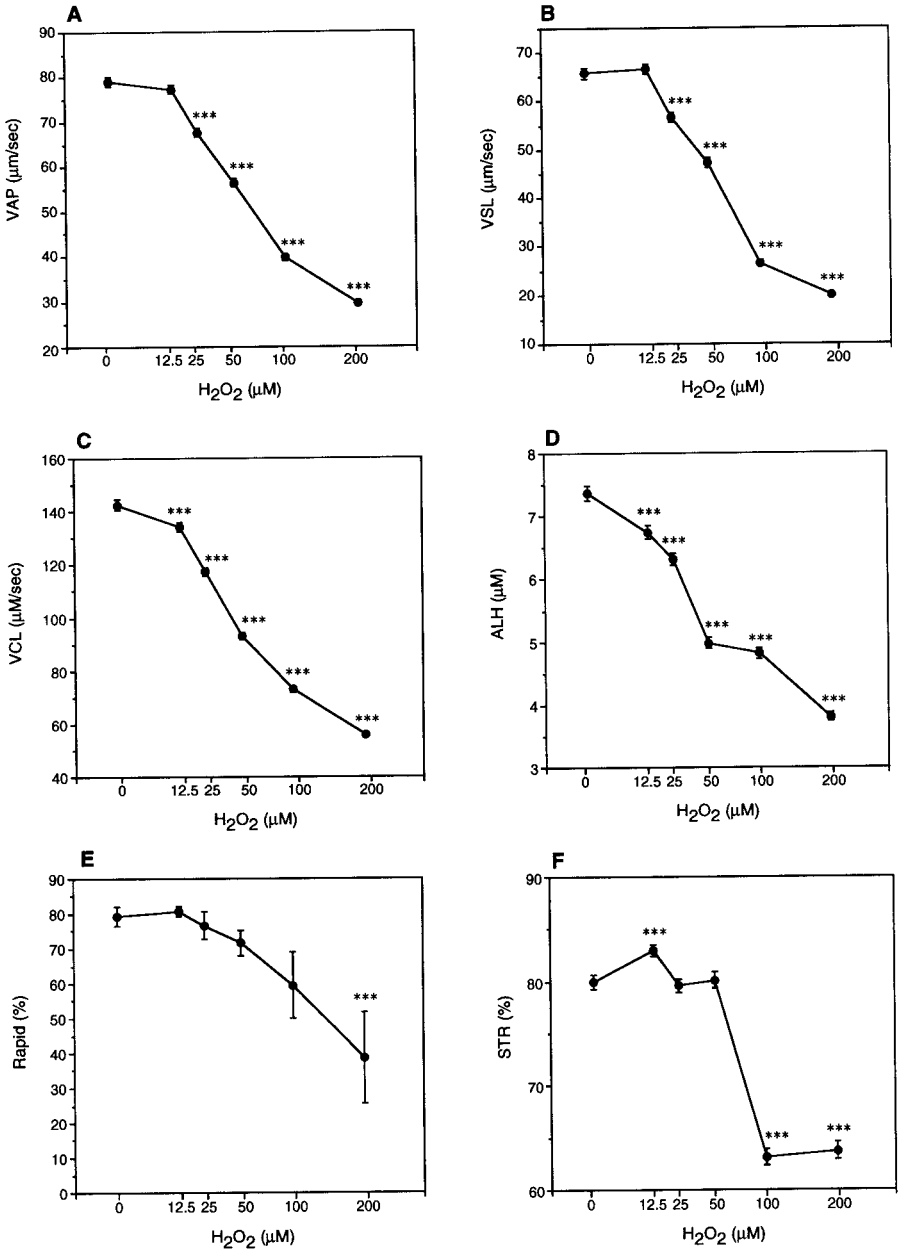

FIG. 7. Analysis of the quality of sperm movement in the face of increasing concentrations of H_2O_2. The movement characteristics illustrated are **A**) VAP, **B**) VSL, **C**) VCL, **D**) ALH, **E**) percentage rapid, **F**) STR. $^{***}p < 0.001$ for 5 independent donor samples. A square-root transformation was used to ensure the even distribution of data points along the x-axis.

excess of 120 μm/sec (Figs. 3 and 7). The implication of these findings—that spermatozoa suffering from an intermediate level of oxidative stress and possessing damaged DNA are nevertheless capable of fertilization—is borne out by clinical evidence revealing a chain of associations between oxidative stress in the ejaculate, DNA damage to the spermatozoa, and elevated levels of childhood cancer in the offspring [30, 55, 56]. This evidence is based on the observation that the oxidative stress associated with heavy smoking suppresses the levels of antioxidants present in both serum (vitamin C) and semen (vitamin E) and results in the appearance of oxidative damage to the sperm DNA [30]. That such oxidative damage to the sperm genome is associated with mutations in the male germ line that are passed on to the offspring at fertilization, is emphasized by two recent independent studies indicating that the children of heavy smokers exhibit a significantly increased risk of developing childhood cancer [55, 56].

The highest levels of oxidative stress, achieved by incubating human spermatozoa in the presence of 10–20 mM NADPH or 50–200 μM H_2O_2, were associated with a dramatic decline in the functional and genomic integrity of the spermatozoa. Such results emphasize the inherent vulnerability of the male germ cell to oxidative stress, particularly during late spermatogenesis and the early stages of epididymal maturation. At this point in the life history of these cells, the DNA repair mechanisms have been turned off, the reduced cytoplasmic volume has limited their antioxidant status, the DNA is not fully compacted, and they express an active capacity for free radical generation. As a consequence of this vulnerability, exposure to ROS can rapidly overwhelm the spermatozoon's defenses and induce DNA damage, such that at the highest levels of oxidative stress examined in this study, more than 75% of the DNA was fragmented. Under physiological conditions such severely damaged spermatozoa could not participate in fertilization because collateral peroxidative damage to the sperm plasma membrane would seriously impair the capac-

ity of these cells for movement and sperm-oocyte fusion [20]. Thus in the present study, exposure of the spermatozoa to the highest levels of oxidative stress resulted in a sudden collapse in both the quality of sperm movement and the rates of sperm-oocyte fusion (Figs. 2, 3, 6, and 7). Of these two aspects of sperm function, movement appeared to be more sensitive to the detrimental effects of oxidative stress, since several CASA parameters were disrupted at levels of oxidative stress (5 mM NADPH and 25 μM H_2O_2) at which the rates of sperm-oocyte fusion remained elevated. Indeed, simple and stepwise linear regression analysis revealed an extremely tight correlation between DNA damage and the quality of sperm movement (Fig. 5) under the experimental conditions employed in this study. In light of such data, the existence of similar correlations between oxidative stress, the detailed attributes of sperm movement, and DNA damage should be examined in the patient population.

The concomitant loss of genomic integrity and functional competence seen at the highest levels of oxidative stress represents a biological safeguard designed to ensure that spermatozoa possessing highly fragmented DNA are unlikely to participate in the process of conception. However, the chances of such cells fertilizing the oocyte would clearly be enhanced if the collateral damage to the sperm plasma membrane were circumvented through the use of intracytoplasmic sperm injection (ICSI). Recent studies have demonstrated that spermatozoa exposed to high levels of oxidative stress using the strategies outlined in this paper (exposure to H_2O_2 and NADPH) are capable of achieving normal rates of fertilization with ICSI [57]. The fact that ICSI is being used with just the kind of poor-quality, oligoasthenozoospermic ejaculates that are known to suffer oxidative stress [54, 58] suggests that spermatozoa with oxidatively damaged DNA are being used to create human embryos as a result of current clinical practice. Given the association between oxidative DNA damage to human spermatozoa and childhood cancer in the offspring [30, 55], there are rational grounds for expressing concern over the long-term health of children born as a consequence of the ICSI procedure.

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