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Quantitative analysis of gene-specific DNA damage in human spermatozoa

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Abstract

Recent studies have suggested that human spermatozoa are highly susceptible to DNA damage induced by oxidative stress. However, a detailed analysis of the precise nature of this damage and the extent to which it affects the mitochondrial and nuclear genomes has not been reported. To induce DNA damage, human spermatozoa were treated in vitro with hydrogen peroxide (H_2O_2; 0–5 mM) or iron (as $Fe(II)SO_4$, 0–500 μM). Quantitative PCR (QPCR) was used to measure DNA damage in individual nuclear genes (*hprt*, *β-pol* and *β-globin*) and mitochondrial DNA. Single strand breaks were also assessed by alkaline gel electrophoresis. H_2O_2 was found to be genotoxic toward spermatozoa at concentrations as high as 1.25 mM, but DNA damage was not detected in these cells with lower concentrations of H_2O_2. The mitochondrial genome of human spermatozoa was significantly ($P < 0.001$) more susceptible to H_2O_2-induced DNA damage than the nuclear genome. However, both nDNA and mtDNA in human spermatozoa were significantly ($P < 0.001$) more resistant to damage than DNA from a variety of cell lines of germ cell and myoblastoid origin. Interestingly, significant DNA damage was also not detected in human spermatozoa treated with iron. These studies report, for the first time, quantitative measurements of DNA damage in specific genes of male germ cells, and challenge the commonly held belief that human spermatozoa are particularly vulnerable to DNA damage.

Keywords: DNA damage; Human spermatozoa; Oxidative stress; Mitochondrial DNA; Quantitative PCR

1. Introduction

The genetic integrity of the male germline has impacts upon fertility, the progress of pregnancy and, ultimately, the health and well-being of the offspring [1]. Human males contribute heavily to the germ line mutation load, being responsible for a vast majority of dominant genetic diseases and contributing significantly to the etiology of childhood cancer [1,2]. Mutation in the male germ line creates the genetic diversity that supports the process of natural selection. In this sense, the DNA damage that contributes to this process can be regarded as advantageous and the testes seen as an engine of evolution. However, in excess, DNA damage becomes detrimental to species survival by increasing the incidence of genetic abnormalities in offspring. In light of these conflicting, yet significant, biological outcomes, it is imperative that we define

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and understand the susceptibility of the male germ line to genotoxic agents. Oxidative stress has been shown to play an important role in male infertility, and may also cause germ line DNA damage and mutations [2–4]. The testes may experience oxidative stress from a variety of sources. For example, xenobiotics including solvents, heavy metals, cigarette smoke, and polycyclic aromatic hydrocarbons, all known testicular toxicants, appear to act through oxidative mechanisms [5–8]. In addition, infection, lack of antioxidant vitamin intake, and zinc deficiency have all been associated with decreased sperm function and increased oxidative DNA damage in the male germ line [9–12].

Recent studies have shown that reactive oxygen species (ROS) and other pro-oxidant treatments can induce DNA fragmentation in human spermatozoa [13–17]. This is important since these cells appear to intrinsically generate ROS including both superoxide anion and hydrogen peroxide (H_2O_2) [18–20]. In addition, abnormal spermatozoa from subfertile patients appear to produce excessive levels of ROS, which may lead to DNA damage and ultimately germ line mutations [20–25]. Indeed, spermatozoa from subfertile patients tend to contain higher levels of DNA strand breaks and oxidative base damage than sperm from fully fertile males [26–31]. The assays employed for measuring ROS-induced DNA damage in human spermatozoa include the TUNEL assay, the nick translation assay, and the single cell gel electrophoresis (comet) assay [13–17]. These studies have reported that H_2O_2 treatment of human spermatozoa in vitro at concentrations in the 100–200 μM range induce extensive DNA fragmentation. However, these assays measure global DNA damage in whole cells, and a thorough analysis of these DNA fragments, or DNA damage at the gene level, has not been performed. Furthermore, a quantitative measure of DNA damage in individual genes would be of great benefit since mutation rates could be correlated directly with lesion frequencies in important disease genes. In order to address this issue, we have initiated a more detailed characterization of DNA fragmentation induced by such treatments. Toward this goal, we have quantitatively measured gene-specific DNA damage in both nuclear and mitochondrial genomes of human spermatozoa treated with H_2O_2 or exposed to the redox-active metal, iron.

2. Materials and methods

2.1. Preparation and treatment of human spermatozoa

All reagents used in these studies were molecular biology grade. Ejaculates were obtained from the University of Newcastle Donor Panel in accordance with institutional standards and ethics. A total of 30 semen samples were collected from 12 different donors were used in these experiments. Once collected, the ejaculates were allowed to liquefy for 30 min at 37 °C. The semen was then analyzed to determine sperm concentration, motility, morphology, and vitality, after which it was fractionated by 50%/100% discontinuous Percoll gradient centrifugation, as previously described [32]. For these experiments, cells at the 50%/100% interface were discarded; only the 100% fraction was used to ensure the highest quality sperm population available. The 100% fractions from individual ejaculates were collected, pooled, and washed with 10 ml Biggers–Whitten–Whittingham medium (BWW; 94.6 mM NaCl, 4.78 mM KCl, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_2{\cdot}7H_2O$, 1.71 mM $CaCl_2$, 21.58 mM sodium lactate, 0.33 mM sodium pyruvate, 25.07 mM $NaHCO_3$, 5.56 mM glucose) supplemented with 0.3% bovine serum albumin (fraction V) [33]. Cell pellets were resuspended in 500 μl BWW, and the sperm parameters mentioned above were assessed. Motility and vitality were greater than 80 and 90%, respectively, in all the experimental samples. Aliquots of 5×10^6 cells were then treated for 1 h in 1 ml BWW with the indicated compounds in the dark, at room temperature, and with gentle agitation. In additional experiments, human spermatozoa were treated exactly as described above with H_2O_2, but at 37 °C rather than room temperature. H_2O_2 was prepared from a 30% solution as follows. A 1:10 dilution was made with distilled water to obtain a 3% H_2O_2 solution. A 20 μl aliquot of this solution was diluted with 980 μl water to obtain a 15–20 mM solution. The exact concentration of H_2O_2 in this solution was determined by measuring the absorbance at 240 nm in a spectrophotometer. An extinction coefficient of 44 cm^{-1} mol^{-1} was used to calculate the molarity of the H_2O_2 from the absorbance reading. This working stock was then diluted to obtain the final concentrations of H_2O_2 reported in the data figures. For iron treatments, a

20 mM stock solution of Fe_2SO_4 was prepared and diluted appropriately to obtain the final concentrations reported herein. After the treatments, the cells were pelleted by centrifugation, washed twice in ice-cold PBS, and stored at −80 °C. Preliminary experiments determined that iron was not washed away from the cells with PBS alone. This resulted in the artifactual induction of DNA damage by this metal during cell lysis and DNA purification (described below; data not shown). Thus, for the iron treatments, desferrioxamine (5 mM) was included in the PBS and lysis buffer to facilitate removal of residual iron from the cells. Prior to storing at −80 °C, small aliquots of cells in PBS were prepared for analysis by alkaline gel electrophoresis.

2.2. Cell culture and treatments

Two immortalized murine germ cell lines, GC1 and GC2, and the murine myoblastoid cell line C2C12, were cultured in DMEM plus 5% fetal calf serum at 37 °C and 5% CO_2, and were treated with H_2O_2 as described [34–36]. Briefly, the day before the experiments, the media was replaced by phenol red-free DMEM containing 0.5% fetal calf serum and incubated at 37 °C, 5% CO_2 overnight. H_2O_2 was then added to this media to obtain the final concentrations reported herein. After treatment for 1 h, the cells were washed twice with ice-cold PBS, trypsinized, pelleted by centrifugation, and stored at −80 °C.

2.3. Alkaline gel electrophoresis

Alkaline gel electrophoresis was performed as described [37]. Briefly, approximately 1×10^6 cells were resuspended in 1% agarose, and formed into 10 μl gel "buttons". The embedded cells were lysed overnight (2.5 M NaCl, 100 mM Tris [pH = 10.0], 10 mM EDTA, 1% sarcosyl, 1% Triton X-100) at 4 °C. Samples were then treated overnight at 37 °C with 1 mg ml^{-1} proteinase K in lysis buffer, minus the detergents sarcosyl and Triton X-100. Agarose buttons were stored in lysis buffer minus Triton X-100 at 4 °C. To assess alkali-labile sites, the gel buttons were neutralized by three, 5 min washes in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH = 8.0), and then incubated in alkaline loading dye (500 mM NaOH, 50% glycerol, 0.25% bromocresol green) for 30 min at room temperature. The buttons were loaded into a 0.4% agarose gel prepared in 50 mM NaCl, 4 mM EDTA that had been soaked in alkaline running buffer (30 mM NaOH, 2 mM EDTA) overnight at 4 °C. Electrophoresis was carried out for 18 h running at 1.5 V cm^{-1} and 40–50 mA. After electrophoresis, the gel was neutralized and stained with ethidium bromide (1 μg ml^{-1}). DNA was visualized and photographed under ultraviolet transillumination.

2.4. DNA isolation from experimental samples

DNA from all experimental samples was isolated using standard phenol/chloroform extractions. Briefly, cells were resuspended in STE buffer (50 mM NaCl, 10 mM Tris–HCl pH = 8.0, 1 mM EDTA) containing 1% SDS and 0.5 mg ml^{-1} proteinase K. For human spermatozoa, β-mercaptoethanol was included in the lysis buffer at a concentration of 1% (v/v), and proteinase K was used at a final concentration of 2 mg ml^{-1}. Lysis was performed at 55 °C for 6–8 h with gentle agitation. For experiments involving iron, desferrioxamine (5 mM) was included in the lysis buffer to prevent post-lysis DNA damage. The lysates were extracted sequentially with equal volumes of phenol, phenol/chloroform, and chloroform, and DNA was ethanol precipitated overnight at −20 °C. Precipitated DNA was washed with 70% ethanol, resuspended in TE buffer, and quantitated by PicoGreen (Molecular Probes; Eugene, OR, USA) fluorescence using a Bio-Rad (Regent's Park, NSW) fluorescence plate-reader.

2.5. Quantitative PCR

Quantitative PCR (QPCR) was performed as described [38,39]. Briefly, purified DNA was quantitated by PicoGreen fluorescence. Aliquots of DNA (7.5–50 ng) were then subjected to PCR under quantitative conditions. To ensure that the latter pertained, control reactions containing half the concentration of control template DNA were included in each set of PCR reactions. These samples were prepared by diluting control DNA 1:1 with TE buffer. For human spermatozoa, primers specific for an 8.9 kb fragment of the human mitochondrial genome (mtDNA), a 10.4 kb fragment of the *hprt* gene (nuclear), a 12.2 kb fragment of the *β-pol* gene (nuclear), and a 13.5 kb

fragment on the *β-globin* gene (nuclear) were used in the PCR reactions [39]. For GC1, GC2, and C2C12 cell lines, primers specific for a 10 kb fragment of murine mtDNA, and for a 9 kb fragment of the nuclear *β-globin* gene were used in the PCR reactions [39]. The respective genes were chosen based on our ability to reproducibly and consistently amplify the target of interest under quantitative conditions. Details regarding primer sequences and PCR reaction conditions have been published [39]. No differences in the susceptibility of the various nuclear genes were noted during these experiments (data not shown). Thus, data are reported as nuclear DNA damage (nDNA) versus mitochondrial DNA damage (mtDNA). All PCR reactions were performed in Hybaid PCRExpress 96-well thermocyclers. After thermocycling, PCR products were quantitated by PicoGreen fluorescence using a fluorescence microplate reader (Bio-Rad). PCR reaction mixtures (10 μl) were measured in duplicate. PCR reactions containing no template DNA were performed to provide blanks.

2.6. Gel electrophoresis of PCR products

PCR products were resolved by electrophoresis through 0.8% agarose gels containing 1 μg ml^{-1} ethidium bromide. Appropriate volumes of 6× loading buffer (0.25% xylene cyanol, 0.25% bromophenol blue, 50% glycerol) were added directly to PCR reaction mixtures. Aliquots of 20 μl were loaded into the gels. Electrophoresis was carried out for 2 h at 100 V. Lambda-PUC DNA mix (MBI Fermentas) was used for DNA size standards.

2.7. Calculation of lesion frequencies

Lesion frequencies were calculated as previously described [38,39,40,41]. Briefly, amplification of treated samples was normalized to controls to generate an amplification ratio. Assuming a random distribution of lesions along the target amplicon, and employing the Poisson expression $f(x) = e^{-\lambda}\lambda^x/x!$, where λ is equal to the average lesion frequency, lesion frequencies of treated samples were calculated. In order to use this expression to calculate lesion frequencies, the control samples are assumed to have no lesions [38,39]. Thus, control samples were set to $x = 0$, reducing the Poisson expression to $f(0) = e^{-\lambda}$. The lesion frequency per amplicon was then calculated as $\lambda = -\ln(A_D/A_O)$, where A_D/A_O is the ratio of the amplification of the treated samples (A_D) to the amplification of the control samples (A_O). To determine lesions per 10 kb (10^4 base pairs), the λ-value obtained was divided by the amplicon fragment length, and then multiplied by 10. Multiplying this value again by 10 generates lesions per 10^5 base pairs.

2.8. Hydrogen peroxide measurements

H_2O_2 depletion from cellular medium was measured using an Amplex Red kit from Molecular Probes according to the manufacturer's instructions. For spermatozoa, 5×10^6 cells (taken from the 100% Percoll fraction) were resuspended in 1 ml BWW. H_2O_2 was added to the cells at a final concentration of 200 μM. Cell-free controls were also run in parallel with the experimental samples. At the time points indicated in the appropriate figures, 50 μl aliquots were removed from the bulk suspension, and the cells were pelleted by centrifugation. Aliquots of the supernatant (10 μl) were then diluted with 90 μl Amplex Red buffer and added to 100 μl reaction mixture (containing 100 μM Amplex Red reagent and 0.2 U ml^{-1} horseradish peroxidase) in a 96-well plate. Reactions were allowed to proceed for 30 min at room temperature in the dark, and absorbance was read at 550 nm in a Bio-Rad Ultramark spectrophotometer. The protocol for GC2 cells was essentially identical to that described here for spermatozoa with the following exceptions. Approximately 1×10^6 cells were seeded into T25 cell culture flasks and grown overnight at 5% CO_2, 37 °C in DMEM medium containing 5% FCS. This medium was then replaced with phenol red-free DMEM containing 0.5% FCS, and the cells were incubated at 37 °C, 5% CO_2 over night. H_2O_2 was then added to a final concentration of 200 μM. At the time points indicated in the appropriate figures, 10 μl aliquots of medium were removed, diluted with 40 μl Amplex Red buffer, and H_2O_2 in the medium was measured as described for spermatozoa.

2.9. Statistical analyses

QPCR data values are reported as means, normalized to control values, ±S.E.M. Results of the QPCR were analyzed using the paired Student's *t*-test

comparing each treatment group to its respective control. Lesion frequency data were subjected to one- or two-way ANOVA analyses depending on the number of variables tested, with Fisher's PLSD (protected least significant difference) as the post hoc test in assessing differences between group means. For all experiments, a *P*-value of 0.05 or less was considered significant.

3. Results

3.1. Hydrogen peroxide experiments using human spermatozoa

The QPCR assay used in these studies allowed for the measurement DNA damage in individual nuclear genes and the mitochondrial genome. The fundamental premise of this assay is that DNA damage will arrest the progression of the DNA polymerase used in the PCR reactions [38,39]. Thus, DNA damage is detected as a reduction in the amplification of the target sequence. Experiments were initially performed using two different concentration ranges of H_2O_2 to treat human spermatozoa. Firstly, studies using 0–800 μM H_2O_2 showed no evidence of nuclear DNA damage by QPCR or alkaline gel electrophoresis (data not shown). We did, however, see a slight but significant 17 ± 5% decrease in amplification of the 8.9 kb fragment of mtDNA at concentrations as low as 400 μM (Fig. 1). At 800 μM H_2O_2, amplification of mtDNA was reduced by 20 ± 6% compared to controls (Fig. 1). Based on these observations, we treated human spermatozoa with higher concentrations of H_2O_2 (0–5 mM). Within this concentration range, significant dose-dependent decreases in amplification of both nDNA and mtDNA were observed (Fig. 2). In nuclear genes, a significant 15 ± 2% decrease in amplification was noted after treatment with 1.25 mM H_2O_2, rising to a 38 ± 6% decrease after 5 mM H_2O_2. These decreases in amplification correspond to lesion frequencies of 1.6 and 4.6 per 10^5 base pairs, respectively. As stated above, all nuclear genes were found to have similar susceptibility to DNA damage. Over the same dose range, amplification of an 8.9 kb

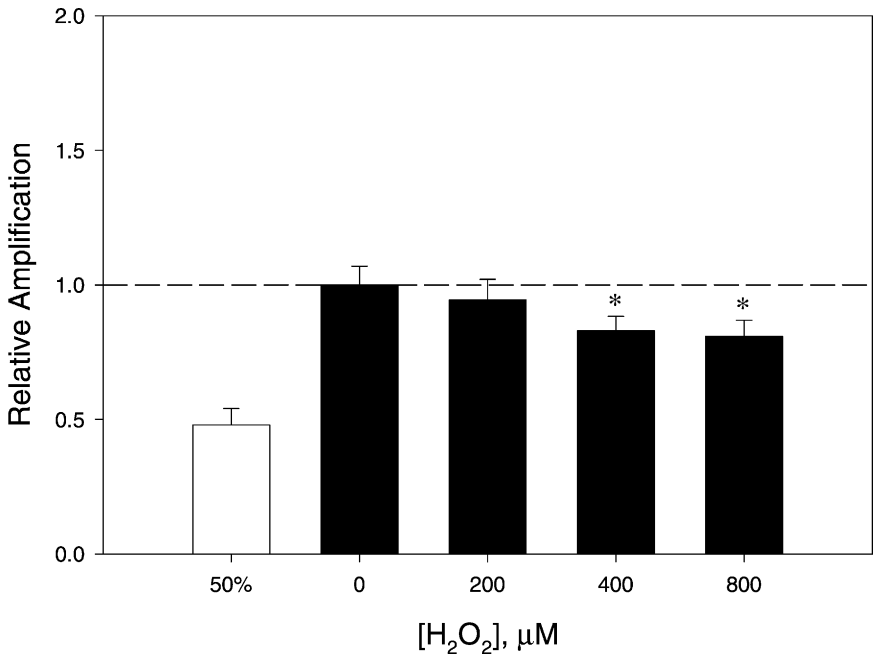

Fig. 1. Effect of low concentrations of hydrogen peroxide on mitochondrial DNA in human spermatozoa. Human spermatozoa were treated for 1 h with the indicated concentrations of hydrogen peroxide. DNA was purified and subjected to quantitative PCR. Raw fluorescence data were normalized to controls, and are presented as mean relative amplification ± S.E.M. from three replicate experiments. $^*P < 0.05$.

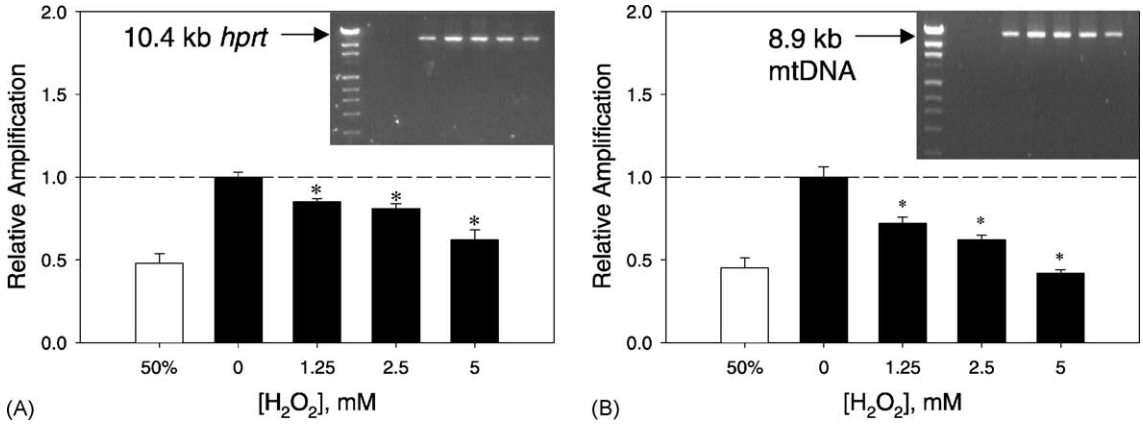

Fig. 2. Effect of hydrogen peroxide on the *hprt* gene and mtDNA. Human spermatozoa were treated for 1 h at room temperature with the indicated concentrations of hydrogen peroxide. DNA was purified and subjected to quantitative PCR. (A) 10.4 kb *hprt*; (B) 8.9 kb mtDNA. Data are presented as described in Fig. 1. The "50%" data represent amplification of 1:1 dilution of control DNA with TE to demonstrate PCRs were carried out under quantitative conditions. The insets are representative gels showing resolved amplicons of the correct size and specificity. Lane 1: Lambda-PUC DNA size marker; Lane 2: empty; Lane 3: PCR reaction blank; Lanes 4–8: PCR reactions as indicated, corresponding to graphed data. $^{*}P < 0.01$. Similar results were obtained for other nuclear genes.

fragment of mtDNA was also significantly reduced in a dose-dependent manner (Fig. 2). Based on Figs. 1 and 2, mtDNA in human spermatozoa appears to be slightly more susceptible to oxidative damage than nDNA. Thus, exposure to 1.25 mM H_2O_2 resulted in 27 ± 2% decrease in amplification for mtDNA, compared with 15 ± 2% for a nuclear gene (*hprt*). Similarly, exposure to 5 mM H_2O_2 reduced mtDNA amplification by 55 ± 1% compared with 38 ± 6% for *hprt*. As will be discussed later, this apparent difference in susceptibility between nuclear and mitochondrial DNA becomes particularly obvious when lesion frequencies are examined on a per kilobase pair basis. It should also be noted that data are included in the figures to demonstrate that both quantitative conditions and amplicon specificity were achieved in these PCR analyses. For each PCR assay, control samples were diluted 1:1 as described in Section 2. This "50%" reaction produced amplification signals that ranged from 43% to 55% of the control samples for all data used in these analyses, averaging 49 ± 6%. In addition, the insets in the figures demonstrate that the target sequence was successfully amplified based on its expected size, and that no non-specific products interfered with the analysis (Figs. 2 and 3).

These results clearly indicated that, contrary to previous reports, human spermatozoa are not highly susceptible to DNA damage after H_2O_2 exposure. In order to assure that this was not a reflection of the fact that the experiments reported here were carried out at room temperature, spermatozoa were also treated with H_2O_2 for 1 h at 37 °C (Fig. 3). It appears that under these conditions, DNA damage was enhanced over that produced by equimolar treatments at room temperature. For example, amplification of nDNA was reduced by 41 ± 13% after treatment with 1.25 mM H_2O_2 at 37 C, but only by 15 ± 2% at room temperature. At 5 mM H_2O_2, amplification of nDNA was reduced by 74 ± 5% at 37 °C, but only by 38 ± 6% at room temperature. However, the susceptibility of these cells to DNA damage did not appear to increase at lower concentrations of H_2O_2 since even at 37 °C, DNA damage was not detected after treatment with 250 μM H_2O_2 (Fig. 3).

To determine if the H_2O_2-induced DNA damage found in individual genes was due to strand breaks, cellular DNA was analyzed by alkaline gel electrophoresis (Fig. 4). In control samples, very high molecular weight DNA ran through the gel matrix as a cohesive mass (Fig. 4). In Fig. 4, the top DNA size marker is 48.5 kb; the size of the control DNA is clearly much larger. We estimate that this DNA is >100 kb in size. With increasing H_2O_2 concentrations, however, the DNA clearly became fragmented, with

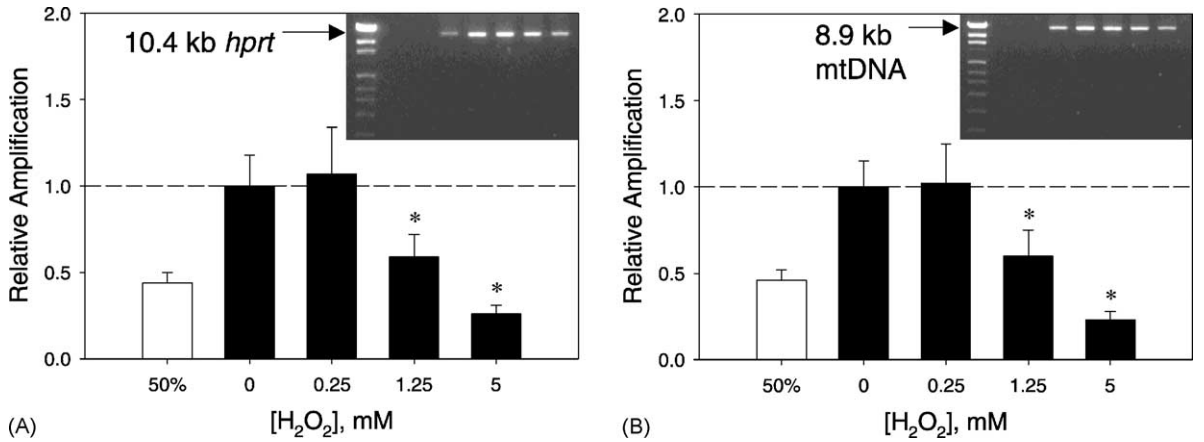

Fig. 3. Effect of hydrogen peroxide treatment of human spermatozoa at 37 °C. Human spermatozoa were treated for 1 h at 37 °C with the indicated concentrations of hydrogen peroxide. DNA was purified and subjected to quantitative PCR. (A) 10.4 kb *hprt*; (B) 8.9 kb mtDNA. Data are presented as described in Figs. 1 and 2. $^{*}P < 0.01$. Similar results were obtained for other nuclear genes.

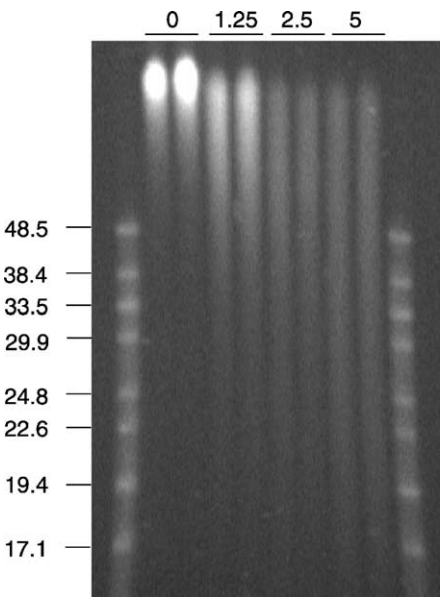

Fig. 4. Analysis by alkaline gel electrophoresis of DNA damage induced by H_2O_2 in human spermatozoa. Human spermatozoa were treated for 1 h at room temperature with 0, 1.25, 2.5, or 5 mM H_2O_2 as indicated in the figure. Cells were then embedded in agarose, lysed, and electrophoresed under alkaline conditions. After electrophoresis, the gel was neutralized, stained with ethidium bromide, and photographed under UV transillumination.

size distributions of DNA fragments shifting toward smaller molecular weight species. In cells treated with 1.25 mM H_2O_2, fragments were observed in the 38–48 kb range, although much of the DNA was still high molecular weight. Higher concentrations induced even more extensive fragmentation, with fragments detected down around the 20–25 kb range after treatment with 5 mM H_2O_2.

3.2. Iron experiments

In order to determine if the resistance of human spermatozoa to H_2O_2 extended to other forms of oxidative stress, we examined the genotoxic effects of iron on these cells. Iron has been shown to damage the DNA of male germ cells in vitro and in vivo [8]. However, as can be seen in Fig. 5, no DNA damage was detected in either nDNA or mtDNA after treatment of human spermatozoa with up to 500 μM Fe(II)SO_4.

3.3. A comparison of lesion frequencies in different cell types

In this study, we sought to compare the relative susceptibilities of different cell types to the DNA damaging effects of H_2O_2. Assuming a random distribution of lesions along the target sequence, and employing the Poisson expression, lesion frequencies can be

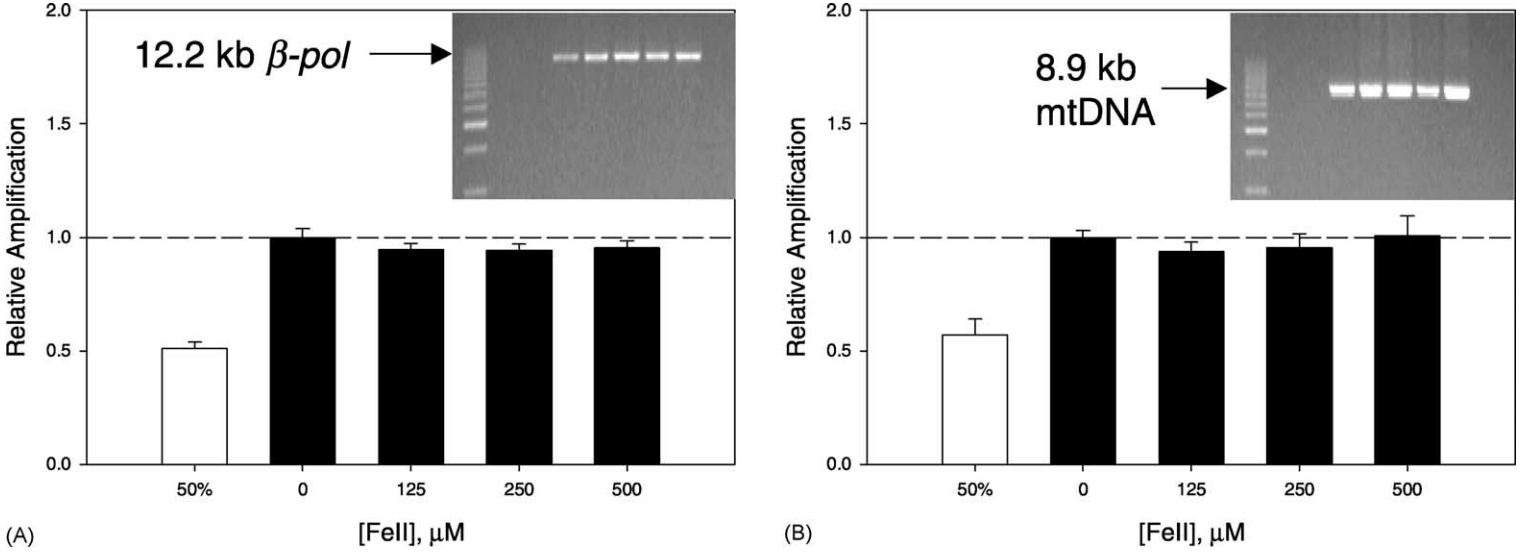

Fig. 5. Effect of iron on the *β-pol* gene and mtDNA. Human spermatozoa were treated for 1 h with the indicated concentrations of iron as $Fe(II)SO_4$. DNA was purified and subjected to quantitative PCR. (A) 12.2 kb *β-pol*; (B) 8.9 kb mtDNA. Data are presented as described in Fig. 2. Similar results were obtained for other nuclear genes.

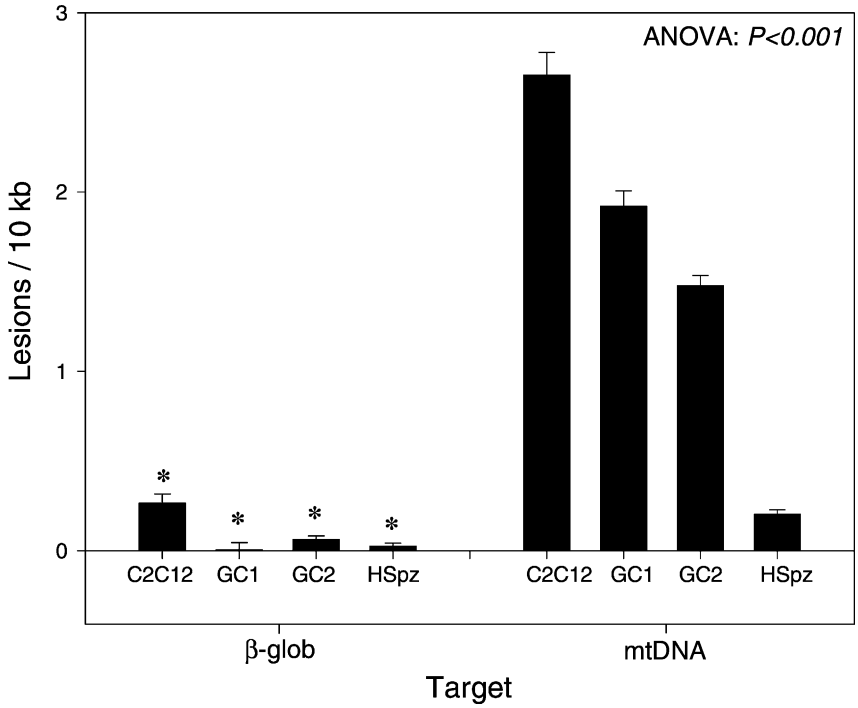

Fig. 6. A comparison of lesion frequencies in different cell types. The myoblastoid cell line C2C12, the immortalized germ cell lines GC1 and GC2, and human spermatozoa were treated for 1 h with 400 μM H_2O_2. Lesion frequencies were calculated as described in Section 2. Lesion frequencies in the germ cell lines were calculated from data published in reference [35].

calculated on a per kilobase pair basis [38–41]. Fig. 6 summarizes the lesion frequencies observed in human spermatozoa compared with three cell lines including two immortalized germ cell lines, GC1 and GC2, and the myoblastoid cell line C2C12. The response of the cell lines to the DNA damaging effects of H_2O_2 was very similar. Little to no damage was seen in nuclear genes in cells (including human spermatozoa) treated with up to 400 μM H_2O_2 (Fig. 6). Higher concentrations were avoided to prevent cytotoxicity or apoptosis, which could have confounded the DNA damage results. This concern was not worrisome regarding spermatozoa since these cells lack the capability of undergoing any form of programmed cell death. The C2C12 cell line did exhibit a significantly increased level of DNA damage in the *β-globin* gene when compared to the GC cell lines and human spermatozoa, but the latter were not significantly different from the other cell lines examined (Fig. 6). In contrast, mtDNA was highly susceptible to oxidative damage in cultured cells, with dramatic dose-dependent increases in lesion frequency observed. In all cultured cells, 400 μM H_2O_2 reduced amplification signals by >90%, resulting in lesion frequencies of 2–3 lesions per 10 kb (Fig. 6). Interestingly, both nDNA and mtDNA in human spermatozoa appeared to be much more resistant to the DNA damaging effects of H_2O_2 than the cultured cells. Lesion frequencies of less than 1 lesion per 10 kb were seen in mtDNA of human spermatozoa treated with 5 mM H_2O_2 (Fig. 7), compared to the 2–3 lesions per 10 kb found in mtDNA of cultured cells treated with 400 μM H_2O_2. Similarly, nuclear genes in human spermatozoa suffered approximately 0.25 lesions per 10 kb after treatment with 400 μM H_2O_2 (not significantly different from controls) versus up to 2.5 lesions per 10 kb in cultured cells, a 10-fold increase.

3.4. Hydrogen peroxide depletion from cellular medium

In an effort to understand the clear differences in the genotoxicity of H_2O_2 toward spermatozoa and

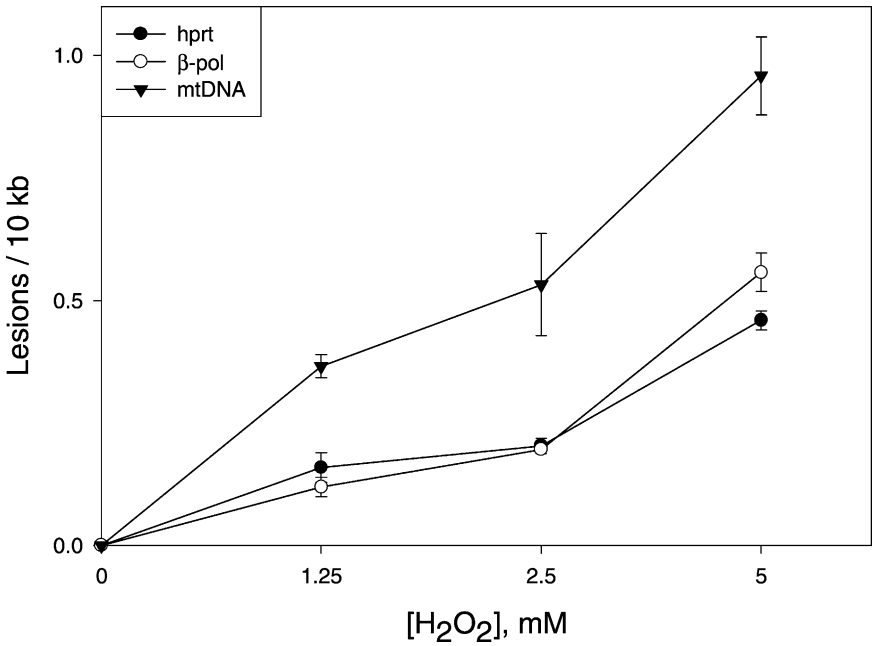

Fig. 7. A comparison of lesion frequencies induced by H_2O_2 in human spermatozoa. Human spermatozoa were treated for 1 h with the indicated concentrations of H_2O_2. Lesion frequencies were calculated as described in Section 2.

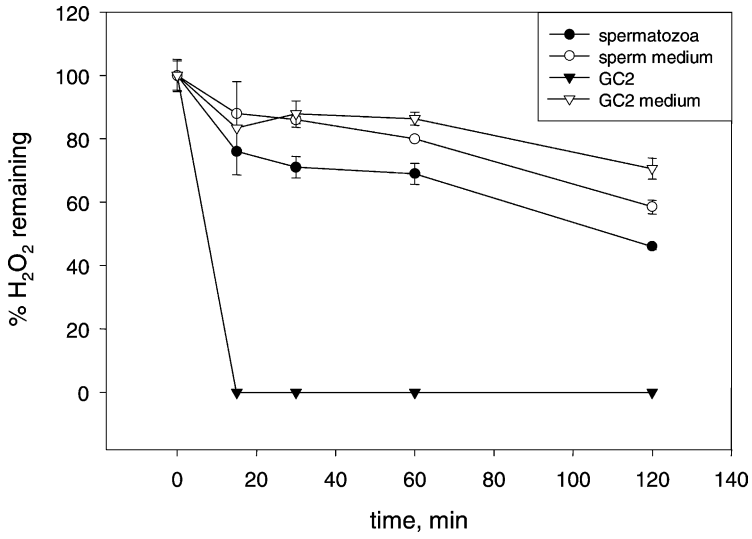

Fig. 8. H_2O_2 decay in the presence of human spermatozoa and GC2 cells. H_2O_2 was added to the respective cells types at a final concentration of 200 μM. Aliquots of cellular medium were removed at the indicated time points and H_2O_2 levels were measured using an Amplex Red kit. The labels "sperm medium" and "GC2 medium" represent cell-free controls ran in parallel with the experimental samples. Data are presented as percent H_2O_2 remaining ± S.D.

cultured cells, particularly in the mtDNA, H_2O_2 depletion from cellular medium was investigated. Spermatozoa appear to metabolize H_2O_2 rather slowly. As can be seen in Fig. 8, after 2 h, almost 50% of the H_2O_2 remains. Indeed, after 8 h, 20% of the H_2O_2 still remained (data not shown), and only at some point between 8 and 20 h did the H_2O_2 completely disappear from the medium. In contrast, GC2 cells metabolize H_2O_2 very quickly. Within 15 min, levels of H_2O_2 added to the cellular medium were undetectable. This effect was clearly not due to the cellular medium since, in the absence of cells, H_2O_2 persisted, with levels still at around 80% after 2 h.

4. Discussion

We have used QPCR to quantitatively measure, for the first time, gene- and mitochondrial-specific DNA damage in human spermatozoa after treatments with H_2O_2 and iron. The primary advantages of the QPCR assay are that only nanogram quantities of DNA are required, and DNA damage can be assessed in individual genes. Additionally, it's well known that most, if not all, single gene mutations that cause human genetic diseases in offspring arise in the male germ line [1]. Thus, an assay with the ability to quantitatively measure DNA damage in individual genes would be a valuable tool for analyzing the relationship between DNA damage and mutation rates. Using QPCR, we were able to detect as few as 1 lesion per 10^5 base pairs after treatment of cells with H_2O_2. The sensitivity we report here agrees closely with previously published QPCR data. For example, others have reported detection of DNA damage at 1–3 lesions per 10^5 base pairs in a variety of other cell types [38,40,41]. This sensitivity rivals that of other DNA damage assays including alkaline elution, alkaline gel electrophoresis (discussed below), and the comet assay, all of which detect 1 lesion per 10^4–10^6 base pairs. We also have confirmed the ability of the QPCR assay to detect DNA damage induced by H_2O_2, as has been reported by others [36,38,41]. This compound induces DNA strand breaks and oxidizes all four of the naturally occurring DNA bases to generate various products recognized as DNA damage [44]. Iron also causes oxidative DNA damage of a sort similar to that of H_2O_2, and thus would be expected to generate QPCR-sensitive lesions.

Using QPCR, we observed a significant increase in DNA damage in nuclear genes after treatment of human spermatozoa with 1.25 mM H_2O_2. Higher concentrations resulted in a corresponding further increase in DNA damage at the gene level. In human spermatozoa, mtDNA appeared to be slightly more susceptible to H_2O_2-induced DNA damage than nDNA, as has been shown in other cell types and tissues [42]. However, when compared to cultured cells, mtDNA in human spermatozoa appeared to be relatively resistant to the DNA damaging effects of H_2O_2. In contrast the mtDNA of cultured germ cells was highly sensitive to the damaging effects of H_2O_2. If this is true in vivo, mtDNA may provide a sensitive biomarker for exposure of germ line DNA to genotoxic agents. Since we did not detect DNA damage in human spermatozoa exposed to micromolar concentrations of H_2O_2 as has been found by others, we repeated these experiments at 37 °C rather than at room temperature. The severity of the DNA damage appeared to increase slightly in response to this treatment regime. However, we were still unable to detect DNA damage using micromolar concentrations of H_2O_2 (compare Figs. 2 and 3).

Using alkaline gel electrophoresis, we have shown that H_2O_2 in millimolar concentrations induces single strand breaks and/or alkali-labile sites in human spermatozoa. With alkaline gel electrophoresis, the DNA backbone is cleaved at alkali-labile sites, which results in a smearing of the DNA fragments in the gel; undamaged DNA runs as a high molecular weight band [37]. With increasing H_2O_2 concentrations, the DNA became increasingly fragmented. These data correlate well with the QPCR data in that the severity of smearing corresponded to the observed decrease in amplification over the same concentration range. The alkaline gel electrophoresis assay also provides another sensitive endpoint for measuring DNA damage in cell populations. This assay has been reported to detect $\sim$2 lesions per 10^6 base pairs, an order of magnitude greater sensitivity than the QPCR assay [37]. Thus, both assays used in this study for measuring DNA damage are sensitive to 1 lesion per 10^5–10^6 base pairs, and agree closely with each other with regard to the effect of H_2O_2 on sperm DNA.

The results obtained in the cultured cell experiments suggest that nuclear DNA of the cell lines GC1, GC2

and C2C12 are also relatively resistant to the induction of DNA damage by H_2O_2. In contrast, a transformed human fibroblast appears to be very susceptible to the DNA damaging effects of H_2O_2. Yakes and Van Houten [38] reported that 100–200 μM H_2O_2-induced significant nuclear DNA damage at the gene level in these cells (∼0.5–1 lesions per 10 kb). Moreover, this finding has since been independently verified [43]. Thus, it appears that some cells are much more susceptible than others to the genotoxic effects of H_2O_2. In particular, human spermatozoa and immortalized murine germ cells appear to be relatively resistant to H_2O_2. In contrast to nDNA, mtDNA of cultured cells was highly susceptible to the DNA-damaging effects of H_2O_2 as has been shown in other cell lines and animal tissues [42]. In these cells, mtDNA suffered 2–3 lesions per 10 kb, while no damage was observed in nDNA.

The apparent resistance of human sperm DNA to damage by H_2O_2 found in this study conflicts somewhat with other reports. For example, it has been reported that extensive DNA damage is induced by H_2O_2 in the micromolar concentration range [13–17]. It may be that the assays employed in those reports (e.g. TUNEL, comet) are more sensitive than the ones used here, although calculations of lesion frequencies in this study do not support this contention. Problems with the sensitivity and specificity of these assays, particularly the TUNEL assay, have been noted [45]. It also remains to be seen what the mutagenic potential will be of the relative amounts of DNA damage detected by these different assays. As mentioned above, quantitative measurements of DNA damage in individual disease genes would be invaluable for obtaining quantitative relationships between DNA damage and resulting mutation rates. Simply stated, with assays such as QPCR, we may better be able to answer the question, “how much DNA damage is needed to generate a specific mutation rate?” In support of the argument that sperm DNA is relatively resistant to induced oxidative damage, we found that the redox-active metal, iron, also did not induce detectable DNA damage in human spermatozoa.

Interestingly, other reports agree closely with the findings of our study in regard to other oxidative and biological endpoints in human spermatozoa. For example, millimolar concentrations of H_2O_2 are required to induce lipid peroxidation and loss of motility in human spermatozoa [46]. Another report has shown that mitochondrial membrane potential is decreased by ∼70% after treatment with 5 mM H_2O_2, while 20 mM H_2O_2 was required to effect a 94% decrease in the same parameter [47]. These data correlate well with the observed 50% decrease in amplification of mtDNA reported here in human sperm treated with 5 mM H_2O_2. Clearly, the concentrations of H_2O_2 mentioned here are non-physiological. Thus, it appears that sperm DNA would be protected from the small amount of H_2O_2 they might generate under natural circumstances [4].

Further evidence of the resistance of human spermatozoa to chemical and physical agents has been provided in other reports. For example, Twigg et al. [15], have shown that human spermatozoa treated with 1 mM H_2O_2 for 1 h at 37 °C were still able to form normal pronuclei after injection into hamster oocytes. The studies of Ahmadi and Ng [48,49] provide clues as to how far an embryo generated from such a heavily damaged male gamete might progress in terms of development. Their results suggest that rather intense chemical and physical treatment is required to adversely influence the development of mammalian embryos. For example, human spermatozoa exposed to 100 Gy γ-irradiation were still able to penetrate hamster eggs. Mouse spermatozoa exposed in the same manner generated two-cell embryos, but blastocyst formation, the number of implantation sites, and the number of live fetuses, were all significantly reduced. Thus, it appears that the mature spermatozoon is quite resistant to chemical and physical damage. Indeed, these cells have spore-like qualities with a highly compacted, genetically quiescent genome, which, only under the correct environmental conditions, becomes an active template for the continuation of the life cycle.

H_2O_2 decay in the presence of spermatozoa was shown to be quite slow. Fig. 8 shows that about one-half of the H_2O_2 initially added to human spermatozoa still remains after 2 h. In contrast, GC2 cells metabolize all of the H_2O_2 added within 15 min. This is to be expected since these cells have much larger volumes of antioxidant-rich cytoplasm with which to break down H_2O_2. Spermatozoa, in contrast, have very little cytoplasm and few antioxidant capabilities other than the selenium-dependent glutathione peroxidase [50]. Interestingly, this enzyme localizes to the mitochondria, which may afford the mtDNA

some protection. Furthermore, the sperm-specific glutathione peroxidase appears to utilize lipid hydroperoxides as substrates much more efficiently than H_2O_2, although the latter appears to also be metabolized by this enzyme [50]. Thus, whatever the reason sperm mtDNA is so resistant to H_2O_2, it is clearly not due to these cells' ability to metabolize and break down H_2O_2 quickly.

In summary, these data demonstrate that: (i) measurement of gene-specific DNA damage is possible in human spermatozoa and other germ cells using QPCR, (ii) nuclear DNA of human spermatozoa is relatively resistant to damage after H_2O_2 and iron treatment in vitro, (iii) sperm mtDNA, while resistant to damage compared with other cell types, may serve as a sensitive biomarker of oxidative stress in male germ cells. The QPCR assay, due to its gene-specific nature, may also prove useful for "scanning" the genome for DNA damage hotspots. Such information would be valuable, in particular, to those seeking assisted reproduction therapy for the treatment of male infertility.

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References

[1] R.J. Aitken, The human spermatozoon—a cell in crisis? (The Amoroso Lecture), J. Reprod. Fertil. 115 (1999) 1–7.
[2] J.F. Crow, The origins, Nat. Rev. Genet. 1 (2000) 40–47.
[3] J.F. Griveau, D. Le Lannou, Reactive oxygen species and human spermatozoa: physiology and pathology, Int. J. Androl. 20 (1997) 61–69.
[4] B.N. Ames, M.K. Shigenaga, T.M. Hagen, Oxidants, antioxidants, and the degenerative diseases of aging, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 7915–7922.
[5] C.G. Fraga, P.A. Motchnik, A.J. Wyrobeck, D.M. Rempel, B.N. Ames, Smoking and low antioxidant levels increase oxidative damage to sperm DNA, Mutat. Res. 351 (1996) 199–203.
[6] P.G. Wells, P.M. Kim, R.R. Laposa, C.J. Nicol, T. Parman, L.M. Winn, Oxidative damage in chemical teratogenesis, Mutat. Res. 396 (1997) 65–78.
[7] M. Ishihara, M. Itoh, K. Mayamoto, S. Suna, Y. Takeuchi, I. Takenaka, F. Jitsunari, Spermatogenic disturbance induced by di-(2-ethylhexyl) phthalate is significantly prevented by treatment with antioxidant vitamins in the rat, Int. J. Androl. 23 (2000) 85–94.
[8] A. Wellejus, H.E. Poulsen, S. Loft, Iron-induced oxidative DNA damage in rat sperm cells in vivo and in vitro, Free Rad. Res. 32 (2000) 75–83.
[9] C.G. Fraga, P.A. Motchnik, M.K. Shigenaga, H.J. Helbock, R.A. Jacob, B.N. Ames, Ascorbic acid protects against endogenous oxidative DNA damage in human sperm, Proc. Natl. Acad. Sci. U.S.A. 88 (1991) 11003–11006.
[10] P.I. Oteiza, K.L. Olin, C.G. Fraga, C.L. Keen, Zinc deficiency causes oxidative damage to proteins, lipids and DNA in rat testes, J. Nutr. 125 (1995) 823–829.
[11] F.R. Ochsendorf, Infection and reactive oxygen species, Andrologia 30 (Suppl. 1) (1998) 81–86.
[12] F.H. Comhaire, A.M. Mahmoud, C.E. Depuydt, A.A. Zalata, A.B. Christophe, Mechanisms and effects of male genital tract infection on sperm quality and fertilizing potential: the andrologists viewpoint, Hum. Reprod. Update 5 (1999) 393–398.
[13] C.M. Hughes, S.E. Lewis, V.J. McKelvey-Martin, W. Thompson, A comparison of baseline and induced DNA damage in human spermatozoa from fertile and infertile men, using a modified comet assay, Mol. Hum. Reprod. 2 (1996) 613–619.
[14] R.J. Aitken, E. Gordon, D. Harkiss, J.P. Twigg, P. Milne, Z. Jennings, D.S. Irvine, Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa, Biol. Reprod. 59 (1998) 1037–1046.
[15] J.P. Twigg, D.S. Irvine, R.J. Aitken, Oxidative damage to DNA in human spermatozoa does not preclude pronucleus formation at intracytoplasmic sperm injection, Hum. Reprod. 13 (1998) 1864–1871.
[16] E.T. Donnelly, N. McClure, S.E. Lewis, The effect of ascorbate and α-tocopherol supplementation in vitro on DNA integrity and hydrogen peroxide-induced DNA damage in human spermatozoa, Mutagenesis 14 (1999) 505–512.
[17] D.N. Kemal, M. Morshedi, S. Oehninger, Effects of hydrogen peroxide on DNA and plasma membrane integrity of human spermatozoa, Fertil. Steril. 74 (2000) 1200–1207.
[18] J.G. Alvarez, J.C. Touchstone, L. Blasco, B.T. Storey, Spontaneous lipid peroxidation and production of hydrogen peroxide and superoxide in human spermatozoa, J. Androl. 8 (1987) 338–348.
[19] R.J. Aitken, J.S. Clarkson, Generation of reactive oxygen species by human spermatozoa, in: T. Dormandy, C. Rice-Evans (Eds.), Free Radicals: Recent Developments in Lipid Chemistry, Experimental Pathology and Medicine, Richelieu Press, London, 1987, pp. 333–335.
[20] R.J. Aitken, J.S. Clarkson, Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa, J. Reprod. Fertil. 81 (1987) 459–469.
[21] R.J. Aitken, J.S. Clarkson, T.B. Hargreave, D.S. Irvine, F.C. Wu, Analysis of the relationship between defective sperm

function and the generation of reactive oxygen species in cases of oligozoospermia, J. Androl. 10 (1989) 214–220.
[22] R.J. Aitken, D.S. Irvine, F.C. Wu, Prospective analysis of sperm–oocyte fusion and reactive oxygen species generation as criteria for the diagnosis of infertility, Am. J. Obstet. Gynecol. 164 (1991) 542–551.
[23] R. D'Agata, E. Vicari, M.L. Moncada, G. Sidoti, A.E. Calogero, M.C. Fornito, G. Minacapilli, A. Mongioi, P. Polosa, Generation of reactive oxygen species in subgroups of infertile men, Int. J. Androl. 13 (1990) 344–351.
[24] A. Iwasaki, C. Gagnon, Formation of reactive oxygen species in spermatozoa of infertile patients, Fertil. Steril. 57 (1992) 409–416.
[25] F.F. Pasqualotto, R.K. Sharma, H. Kobayashi, D.R. Nelson, A.J. Thomas, A. Agarwal, Oxidative stress in normospermic men undergoing infertility evaluation, J. Androl. 22 (2001) 316–322.
[26] J.G. Sun, A. Jurisicova, R.F. Casper, Detection of deoxyribonucleic acid fragmentation in human sperm: correlation with fertilization in vitro, Biol. Reprod. 56 (1997) 602–607.
[27] H. Kodama, Y. Raizo, J. Fukuda, H. Kasai, T. Tanaka, Increased oxidative deoxyribonucleic acid damage in the spermatozoa of infertile male patients, Fertil. Steril. 68 (1997) 519–524.
[28] S. Lopes, J.G. Sun, A. Jurisicova, J. Meriano, R.F. Casper, Sperm deoxyribonucleic acid fragmentation is increased in poor-quality semen samples and correlates with failed fertilization in intracytoplasmic sperm injection, Fertil. Steril. 69 (1998) 528–532.
[29] H.M. Shen, S.E. Chia, C.N. Ong, Evaluation of oxidative DNA damage in human sperm and its association with male infertility, J. Androl. 20 (1999) 718–723.
[30] E. Host, S. Lindenberg, S. Smidt-Jensen, DNA strand breaks in human spermatozoa: correlation with fertilization in vitro in oligozoospermic men and in men with unexplained infertility, Acta Obstet. Gynecol. Scand. 79 (2000) 189–193.
[31] D.S. Irvine, J.P. Twigg, E.L. Gordon, N. Fulton, P.A. Milne, R.J. Aitken, DNA integrity in human spermatozoa: relationships with semen quality, J. Androl. 21 (2000) 33–44.
[32] R.J. Aitken, J.S. Clarkson, Significance of reactive oxygen species and antioxidants in defining the efficacy of sperm preparation techniques, J. Androl. 9 (1988) 367–376.
[33] J.D. Biggers, W.K. Whitten, D.G. Whittingham, The culture of mouse embryos in vitro, in: J.C. Daniel (Ed.), Methods in Mammalian Embryology, Freeman Press, San Francisco, 1971, pp. 86–94.
[34] M.C. Hoffman, S. Narisawa, R.A. Hess, J.L. Millan, Immortalisation of germ cells and somatic testicular cells using the SV40 large T antigen, Exp. Cell Res. 201 (1992) 417–435.
[35] M.C. Hoffman, R.A. Hess, I. Goldberg, J.L. Millan, Immortalised germ cells undergo meiosis in vitro, Proc. Natl. Acad. Sci. U.S.A. 91 (1994) 5533–5537.
[36] D.E. Sawyer, S.D. Roman, R.J. Aitken, Relative susceptibilities of mitochondrial and nuclear DNA damage induced by hydrogen peroxide in two mouse germ cell lines, Redox Rep. 6 (2001) 182–184.
[37] B.M. Sutherland, P.V. Bennett, J.C. Sutherland, DNA damage quantitation by alkaline gel electrophoresis, Methods Mol. Biol. 113 (1999) 183–202.
[38] F.M. Yakes, B. Van Houten, Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 514–519.
[39] S. Ayala-Torres, Y. Chen, T. Svoboda, J. Rosenblatt, B. Van Houten, Analysis of gene-specific DNA damage and repair using quantitative polymerase chain reaction, Methods 22 (2000) 135–147.
[40] F.M. Yakes, Y. Chen, B. Van Houten, PCR-based assays for the detection and quantitation of DNA damage and repair, in: G.P. Pfeifer (Ed.), Technologies for Detection of DNA Damage and Mutations, Plenum Press, New York and London, 1996, pp. 171–184.
[41] J.J. Salazar, B. Van Houten, Preferential mitochondrial DNA injury caused by glucose oxidase as a steady generator of hydrogen peroxide in human fibroblasts, Mutat. Res. 385 (1997) 139–149.
[42] D.E. Sawyer, B. Van Houten, Repair of DNA damage in mitochondria, Mutat. Res. 434 (1999) 161–176.
[43] G.M. Laws, T.R. Skopek, M.V. Reddy, R.D. Storer, W.E. Glaab, Detection of DNA adducts using a quantitative long PCR technique and the fluorogenic 5′ nuclease assay (Taqman), Mutat. Res. 484 (2001) 3–18.
[44] L.J. Marnett, Oxyradicals and DNA damage, Carcinogenesis 21 (2000) 361–370.
[45] F. Labat-Moleur, C. Guillermet, P. Lorimier, C. Robert, S. Lantuefoul, E. Brambilla, A. Negoescu, TUNEL apoptotic cell detection in tissue sections: critical evaluation and improvement, J. Histochem. Cytochem. 46 (1998) 327–334.
[46] J.G. Alvarez, B.T. Storey, Role of glutathione peroxidase in protecting mammalian spermatozoa from loss of motility caused by spontaneous lipid peroxidation, Gamete Res. 23 (1989) 77–90.
[47] J.S. Armstrong, M. Rajasekaran, W. Chamulitrat, P. Gatti, W.J. Hellstrom, S.C. Sikka, Characterization of reactive oxygen species induced effects on human spermatozoa movement and energy metabolism, Free Rad. Biol. Med. 26 (1999) 869–880.
[48] A. Ahmadi, S. Ng, Fertilizing ability of DNA-damaged spermatozoa, J. Exp. Zool. 284 (1999) 696–704.
[49] A. Ahmadi, S. Ng, Developmental capacity of damaged spermatozoa, Hum. Reprod. 14 (1999) 2279–2285.
[50] H. Imai, K. Suzuki, K. Ishizaka, S. Ichinose, H. Oshima, I. Okayasu, K. Emoto, M. Umeda, Y. Nakagawa, Biol. Reprod. 64 (2001) 674–683.