Human Reproduction, Vol.25, No.10 pp. 2415–2426, 2010
Advanced Access publication on August 17, 2010 doi:10.1093/humrep/deq214

human reproduction
ORIGINAL ARTICLE *Andrology*

Analysis of the relationships between oxidative stress, DNA damage and sperm vitality in a patient population: development of diagnostic criteria

R. John Aitken[1,4,*], Geoffry N. De Iuliis[1], Jane M. Finnie[1,2], Andrew Hedges[2], and Robert I. McLachlan[3,5]

[1]ARC Centre of Excellence in Biotechnology and Development, Discipline of Biological Sciences, School of Environmental and Life Sciences, University of Newcastle, Callaghan, NSW, Australia [2]Hunter IVF, New Lambton, NSW, Australia [3]Prince Henry's Institute of Medical Research, Monash Medical Centre, Clayton, Victoria 3186, Australia [4]Hunter Medical Research Institute, John Hunter Hospital, New Lambton, NSW, Australia [5]Monash IVF, Epworth Hospital, Bridge Road, Victoria 3121, Australia

*Correspondence address. E-mail: jaitken@mail.newcastle.edu.au

Submitted on May 12, 2010; resubmitted on May 31, 2010; accepted on June 2, 2010

BACKGROUND: DNA damage in human spermatozoa is known to be associated with a variety of adverse clinical outcomes affecting both reproductive efficiency and the health and wellbeing of the offspring. However, the origin of this damage, its biochemical nature and strategies for its amelioration, still await resolution.

METHODS: Using novel methods to simultaneously assess DNA fragmentation (modified TUNEL assay), DNA-base adduct formation (8-hydroxy-2′-deoxyguanosine [8OHdG]) and cell vitality, spermatozoa from a cohort of 50 assisted conception patients were examined and compared with a group of donors. Receiver operating characteristic (ROC) curve analysis was then used to examine the frequency distribution of the data and to determine optimized thresholds for identifying patients exhibiting abnormally high levels of DNA damage.

RESULTS: 8OHdG formation and DNA fragmentation were highly correlated with each other and frequently associated with cell death. Percoll centrifugation improved sperm quality but, unexpectedly, increased 8OHdG formation in live cells, as did sperm fractionation using Puresperm® gradients. ROC analysis indicated that the frequency distribution of 8OHdG and DNA fragmentation data were significantly different between patients and donors ($P < 0.001$), permitting the development of thresholds that would allow the accurate diagnosis of DNA damage in the male germ line.

CONCLUSION: The aetiology of DNA damage in spermatozoa involves a cascade of changes that progress from the induction of oxidative stress and oxidized DNA base adduct formation to DNA fragmentation and cell death. Preparation of spermatozoa on discontinuous density gradients aggravates the problem by stimulating the formation of 8OHdG in live cells. However, the development of novel methods and optimized thresholds for diagnosing oxidative DNA damage in human spermatozoa should assist in the clinical management of this pathology.

Key words: DNA damage / spermatozoa / oxidative stress / ROC analysis / infertility

Introduction

DNA damage in the male germ line has been linked with a wide variety of pathological outcomes including reduced fertilization rates, impaired preimplantation development, an increased incidence of miscarriage and morbidity in the offspring (Aitken, 1999; Morris *et al.*, 2002; Carrell *et al.*, 2003; Bungum *et al.*, 2004; Seli *et al.*, 2004; Virro *et al.*, 2004; Lewis and Aitken, 2005; Aitken *et al.*, 2009; Zini and Sigman, 2009; Barratt *et al.*, 2010). The origins of this DNA damage are not fully understood, however several lines of evidence suggest that oxidative stress plays a key role in the underlying aetiology. Spermatozoa are sensitive to such stress because they possess limited endogenous antioxidant protection while presenting abundant substrates for free radical attack in the form of unsaturated fatty acids and DNA (Jones *et al.*, 1979; Aitken and Clarkson, 1987; Alvarez *et al.*, 1987; Aitken 1989, 1999; Koppers *et al.*, 2010).

Electron leakage from the sperm mitochondria is thought to constitute the major source of reactive oxygen species (ROS) in these cells (Koppers *et al.*, 2008). When the production of ROS by the sperm

© The Author 2010. Published by Oxford University Press on behalf of the European Society of Human Reproduction and Embryology. All rights reserved.
For Permissions, please email: journals.permissions@oxfordjournals.org

Downloaded from https://academic.oup.com/humrep/article/25/10/2415/2385624 by guest on 19 April 2024

mitochondria is excessive then the gamete's limited endogenous antioxidant defences are rapidly overwhelmed and oxidative damage is induced. The latter is known to induce lipid peroxidation in the spermatozoa, with a resultant loss of fertilizing potential and vitality (Jones et al., 1979; Alvarez et al., 1987; Aitken et al., 1989, 1998). ROS will also induce DNA damage in the sperm nucleus and mitochondria and will do so at levels of oxidative stress that are still compatible with the retention of motility and a capacity for fertilization (Aitken et al., 1998). As a result, spermatozoa carrying oxidatively damaged DNA could make a significant contribution to the increased levels of miscarriage and childhood disease seen in the offspring of men exhibiting high levels of DNA damage in their spermatozoa (Aitken et al., 2009).

Since oxidative DNA damage is relatively common in human spermatozoa (Kodama et al., 1997; De Iuliis et al., 2009) and the clinical consequences of such damage are so severe (Meseguer et al., 2008; Aitken et al., 2009), serious consideration is being given to the use of antioxidants as a prophylactic treatment for men contemplating assisted conception. Although several studies addressing the therapeutic value of antioxidant treatment for male infertility have generated promising results (Kessopoulou et al., 1995; Suleiman et al., 1996; Greco et al., 2005; Tremellen, 2008), none of these studies have selected patients on the basis of 8-hydroxy-2′-deoxyguanosine (8OHdG) formation or used this oxidized DNA base adduct as a criterion for assessing treatment efficacy. As a result, we have little idea how common oxidative DNA damage is in the target patient population or the extent to which such damage is correlated with DNA fragmentation. We also have no idea whether the method of sperm preparation influences oxidative DNA damage or the extent to which such damage is an indirect reflection of cell viability. In this paper we address these questions and, in the process, generate data on the prevalence of sperm DNA damage in the patient population and the thresholds that might be used in applying this criterion for diagnostic purposes.

Materials and Methods

Semen samples

Two study populations were incorporated into this study. The first comprised a cohort of 50 patients undergoing treatment at an assisted conception clinic (Hunter IVF). These samples were unselected and represent a random cross section of the male population attending such clinics, including those with female factor infertility. The second was a cohort of 36 unselected healthy donors to our reproductive research programme, a majority of whom were students of unknown fertility status. Analysis of the conventional semen profiles for these two groups of subjects did not revealed any statistically significant differences (Fig. 1). For example, oligozoospermia (sperm concentration $<20 \times 10^6$/ml) was present in 17% of the patient samples and in 12% of the donors, while asthenozoospermia (motility <40%) was actually more prevalent in the donors (20%) than the patients (9%). In a supplementary analysis, an additional group of six donor samples were also used to examine the impact of Puresperm® fractionation on DNA damage in human spermatozoa. Scientific use of these samples for research purposes was approved by both an Institutional Human Ethics Committee and the State Minister for Health. After at least 48 h abstinence, semen samples were produced by masturbation and collected into sterile sample containers, which were delivered to the laboratory within 1 h of ejaculation.

Sperm preparation

Purification of human spermatozoa was achieved using a 44 and 88% discontinuous Percoll (GE Healthcare, Castle Hill, Australia) centrifugation gradient, as described (Mitchell et al., 2010). After 30 min centrifugation at 600*g* purified spermatozoa were recovered from the base of the 88% Percoll fraction and washed with Biggers, Whitten and Whittingham medium (BWW; Biggers et al., 1971). These cells were then pelleted by centrifugation at 600*g* for a further 15 min and finally resuspended at a concentration of 6×10^6 cell /ml in BWW. Motility was determined in the BWW medium by transferring 10 µl of the same sample onto a pre-warmed slide, overlaying with a coverslip and subsequently scoring the percentage of motile cells using phase contrast optics. Motility was defined as any cell showing signs of flagellar activity and 200 cells were counted. Vitality was assessed using the eosin exclusion test, by 5 µl of sperm suspension being mixed with 5 µl of 0.5% (w/v) eosin on a microscope slide.

In order to determine whether the results of the flow cytometry analyses were profoundly influenced by the method of sperm preparation, unfractionated sperm suspensions were also prepared by three cycles of centrifugation and resuspension (600*g* for 15 min to give the same total duration of centrifugation as the Percoll prepared samples) in medium BWW.

TUNEL assay

DNA fragmentation and vitality were assessed simultaneously using the novel methodology established by Mitchell et al. (2010). For this version of the assay, spermatozoa were incubated for 30 min at 37°C with LIVE/DEAD® Fixable Dead Cell Stain (far red) (Molecular Probes, Eugene, OR). They were then washed 1× with BWW before being incubated with 2 mM DTT for 45 min and fixed in 2% paraformaldehyde for 15 min at 4°C. After fixation, the spermatozoa were centrifuged (500*g* for 5 min) and washed with phosphate-buffered saline (PBS) before being stored in 0.1 M glycine in PBS at 4°C for a maximum of 1 week. The TUNEL assay was subsequently performed and the percentage of labelled cells was analysed by flow cytometry, as previously described (Mitchell et al., 2010). Gates were set based on forward and side scatter, such that only spermatozoa were assessed. Fluorescence was measured upon excitation by a 15 mW argon-ion laser at 488 nm and was paired with emission measurements using 530/30 band pass (green/FL-1) and >670 long pass (far red/FL-3) filters. For each sample, 10 000 events were recorded at a flow rate of 200–300 cells/s. DNA fragmentation was detected by recording the percentage of cells emitting green fluorescence at 515–555 nm using the FL-1 detector. Cell vitality was determined by recording the percentage of cells emitting far red fluorescence at 665 nm using the FL-3 detector. The software used to analyse the data was Cell Quest Pro (BD Biosciences, USA). Results are presented as either a percentage of the entire population that was positive or as a percentage of the entire population that were alive and positive.

8-hydroxy- 2′-deoxyguanosine

The formation of 8OHdG was measured using a specific antibody (Biotrin OxyDNA test Kit, Biotrin International Ltd, Dublin, Ireland) conjugated to fluorescein isothiocyanate. For the positive control, spermatozoa were incubated for 1 h with H_2O_2 (2 mM) and $Cu^{2+}SO_4$ (1 mM) in a final volume of 200 µl BWW. The H_2O_2 concentration was determined by measuring absorbance at 240 nm. Prior to assessment, all samples were incubated for 30 min at 37°C with LIVE/DEAD® Fixable Dead Cell Stain (far red) (Molecular Probes, Eugene, OR). They were then washed 1× with BWW before being incubated with 2 mM DTT for 45 min and

Downloaded from https://academic.oup.com/humrep/article/25/10/2415/2385624 by guest on 19 April 2024

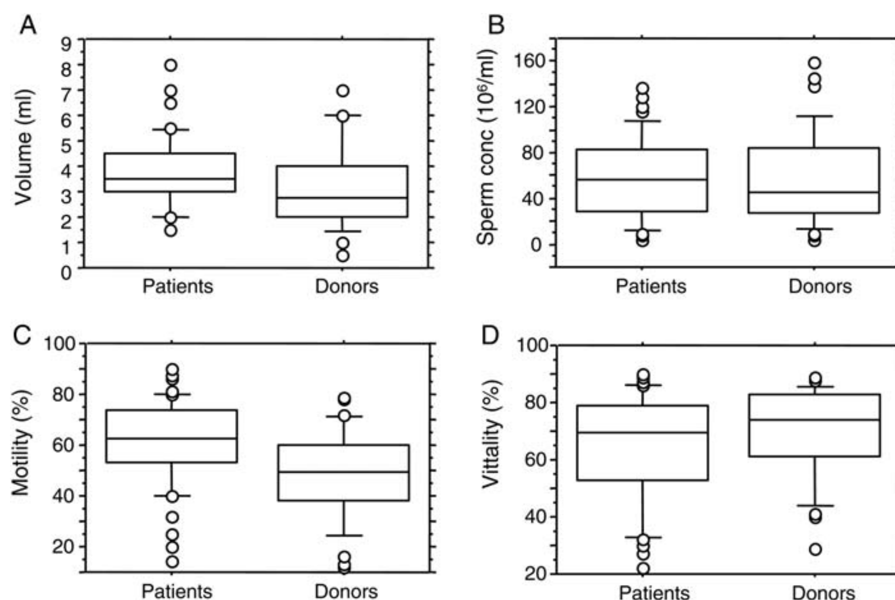

Figure 1 Conventional criteria of semen quality in the patient and donor populations employed in this study. (**A**) Semen volume; (**B**) sperm concentration; (**C**) motility and (**D**) vitality.

fixed in 2% paraformaldehyde for 15 min at 4°C. After fixation, the spermatozoa were centrifuged (500*g* for 5 min) and washed with PBS before being stored in 0.1 M glycine in PBS at 4°C for a maximum of 1 week. The cells were subsequently analysed for 8OHdG expression as previously described (De Iuliis *et al.*, 2009). Fluorescence was then measured on a FACSCalibur flow cytometer (Becton Dickinson) using argon laser excitation at 488 nm coupled with emission measurements using 530/30 band pass (green) using the FL1 channel. Cell vitality was determined by recording the percentage of cells emitting far red fluorescence at 665 nm using the FL-3 detector. Non-sperm-specific events were gated out and 10 000 cells were examined.

Statstical analyses

The results of both the TUNEL and 8OHdG assays were expressed as a percentage of the entire sperm population that were positive irrespective of vitality or as a percentage of the entire population that were alive and positive. Statview (Abacus Concepts Inc, CA, USA) was used for all statistical analyses apart from the receiver operating characteristic (ROC) curve analysis. Paired comparisons were conducted using a paired *t*-test. Non-paired comparisons were conducted with a non-paired *t*-test and confirmed with a non-parametric test (Mann–Whitney *U*). In order to normalize the distribution of the flow cytometry data prior to regression analysis, square root transformation was used. Frequency distribution analyses were also conducted, including calculation of a normal distribution based on each variable's mean and standard deviation. In addition, comparisons of data distributions in the patient and donor samples were conducted by constructing ROC curves using the Xlstat program (Addinsoft, USA; http://www.xlstat.com/en/products/xlstat-life/). The overall significance of either 8OHdG formation or TUNEL positivity as a criterion for distinguishing patients from unselected donors was determined by calculating the area under the ROC curve (AUC). Optimized threshold criteria for distinguishing between these two populations were determined using Youden's index (J = sensitivity + specificity − 1). For all statistical tests, differences with a *P*-value of <0.05% were regarded as significant.

Results

Correlation between DNA fragmentation and 8OHdG formation

The techniques employed in this analysis enabled, for the first time, DNA fragmentation and 8OHdG formation to be analysed in concert with the simultaneous assessment of cell viability in a patient population. When the analysis focused on the viable cells present in unfractionated sperm suspensions, a high level of correlation was observed between DNA fragmentation and 8OHdG formation ($r = 0.671$; $P < 0.001$; Fig. 2A), suggesting that a significant proportion of the DNA damage seen in these cells is oxidatively induced. If these aspects of DNA damage were expressed as percentages of the entire sperm population, irrespective of vitality, then a correlation was still observed but the significance was reduced ($r = 0.355$; $P = 0.011$). The reason for this change in significance may have been due to the confounding influence of cell death, since most of the TUNEL- and 8OHdG-positive cells in these sperm suspensions were found to be non-viable (Fig. 2B). Thus, while 40.1 ± 2.3% of the cells were TUNEL positive only 4.6 ± 0.6% were viable and positive (Fig. 2B) while for 8OhdG, the corresponding figures were 37.3 ± 2.8% and 9.4 ± 1.4%, respectively (Fig. 2C). Within these unfractionated sperm suspensions, vitality was correlated with the outcome of the TUNEL assay ($r = 0.317$; $P = 0.025$) but not with the results of the 8OHdG assay ($r = 0.126$; $P > 0.05$), suggesting that in the cascade of cause and effect leading to DNA damage, 8OHdG is a relatively early event, upstream of DNA fragmentation in the pathway that leads to cell death.

Downloaded from https://academic.oup.com/humrep/article/25/10/2415/2385624 by guest on 19 April 2024

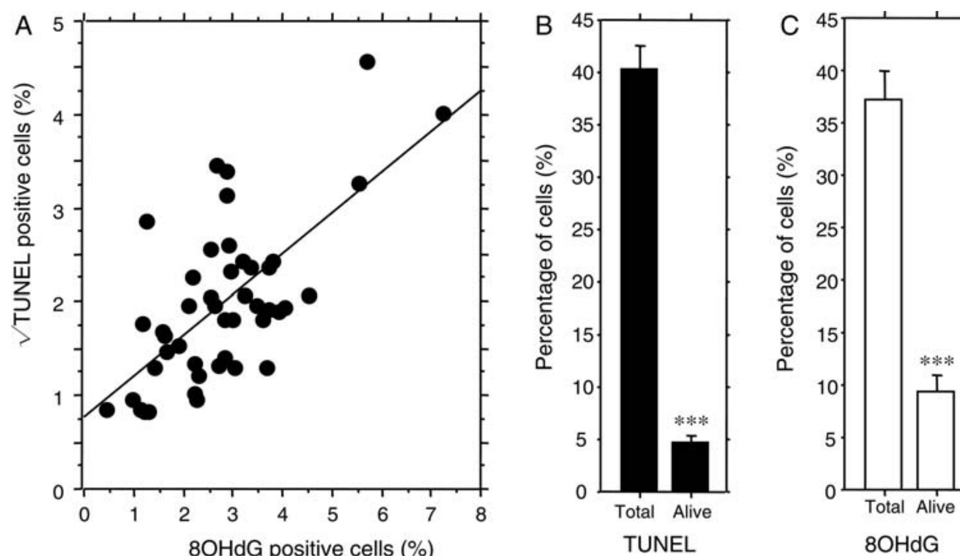

Figure 2 Cell viability, oxidative DNA damage and DNA fragmentation in the patient population ($n = 50$). (**A**) Correlation between 8OHdG formation and DNA fragmentation in unfractionated sperm suspensions. The ordinate and abscissa refer to the percentage of the total sperm population that are alive and positive for either 8OHdG or TUNEL; these data were square root transformed in order to help normalize their distribution. (**B**) Comparison of the total percentage of TUNEL-positive cells and the percentage that are alive and positive. (**C**) Comparison of the total percentage of 8OHdG-positive cells and the percentage that are alive and positive. ***$P < 0.001$ for differences using paired *t*-test.

Impact of Percoll gradient centrifugation

While unfractionated sperm suspensions might be used for diagnostic purposes, the therapeutic use of these cells generally involves fractionated cell populations that have been prepared by discontinuous gradient centrifugation. In order to determine whether the use of such selection methods would impact the incidence of DNA-damaged spermatozoa, the above analyses were repeated on Percoll-prepared spermatozoa. Predictably, Percoll centrifugation significantly improved the percentage of motile and viable cells ($P < 0.001$; Fig. 3A) and in both the unfractionated and Percoll prepared samples, vitality and motility were highly correlated ($P < 0.001$; Fig 3B and C). When the entire sperm population was considered, this improvement in sperm motility and vitality resulting from Percoll fractionation was reflected in a significant decrease in the percentage of TUNEL-positive cells ($P < 0.05$; Fig. 3D), although the percentage of 8OHdG cells remained unchanged (Fig. 3D). Intriguingly, when only the viable cells were considered, Percoll fractionation significantly increased both the percentage of TUNEL- ($P < 0.05$) and 8OHdG-positive cells ($P < 0.001$; Fig. 3E).

In view of the potential significance of this result, we sought to repeat this analysis on an independent data set generated from a cohort of 36 unselected semen donors to our research programme. Within this entire group of 36 samples, Percoll centrifugation again enriched the motility of the sperm suspension and significantly improved vitality ($P < 0.001$; Fig. 4A). Because Percoll centrifugation increased the percentage of viable cells in the sperm suspension, the TUNEL signal for the entire sperm population was significantly reduced ($P < 0.001$; $n = 36$; Fig. 4B), even though Percoll fractionation did not significantly affect the 8OHdG signal ($P > 0.05$; $n = 36$; Fig. 4B). Analysis of cell vitality in relation to DNA damage again revealed that a majority of TUNEL and 8OHdG cells were non-viable both before (Fig. 4C) and after (Fig 4D) Percoll centrifugation. However, if the analysis focused on viable cells, then Percoll centrifugation had little impact on the low incidence of TUNEL -positive live cells in these donor samples, but did significantly increase the percentage of 8OHdG-positive live cells ($P < 0.01$; $n = 36$; Fig. 4E), as observed in the patient population.

These data suggested a stimulatory impact of Percoll centrifugation on 8OHdG formation in an independent data set. In addition, we also examined this effect using discontinuous density gradients created using Puresperm® (colloidal silica particles coated with silane) in an additional group of six donors and the same results were obtained. In this supplementary experiment, preparation of the spermatozoa by discontinuous gradient centrifugation through either Percoll or Puresperm® resulted in significant increases in the proportion of live spermatozoa exhibiting signs of oxidative DNA damage (Percoll, 24.4 ± 4.8% 8OHdG-positive cells; Puresperm®, 26.6 ± 5.1% 8OHdG-positive cells) compared with unfractionated samples prepared by repeated centrifugation in BWW (8.7 ± 1.5% 8OHdG-positive cells; $P \leq 0.01$; $n = 6$). Similarly discontinuous gradient centrifugation through Puresperm® also resulted in a significant increase in the percentage of live spermatozoa exhibiting TUNEL positivity (15.6 ± 5.7%) compared with the unfractionated control samples prepared by repeated centrifugation through BWW (3.7 ± 0.9% TUNEL-positive cells; $P < 0.05$; $n = 6$).

Comparison of the donor and patient populations

Comparison of the TUNEL signals recorded for the donor ($n = 36$) and patient populations ($n = 50$) revealed significant differences, in both unfractionated (donors, 30.9 ± 1.7% versus patients, 40.2 ± 2.3%;

Downloaded from https://academic.oup.com/humrep/article/25/10/2415/2385624 by guest on 19 April 2024

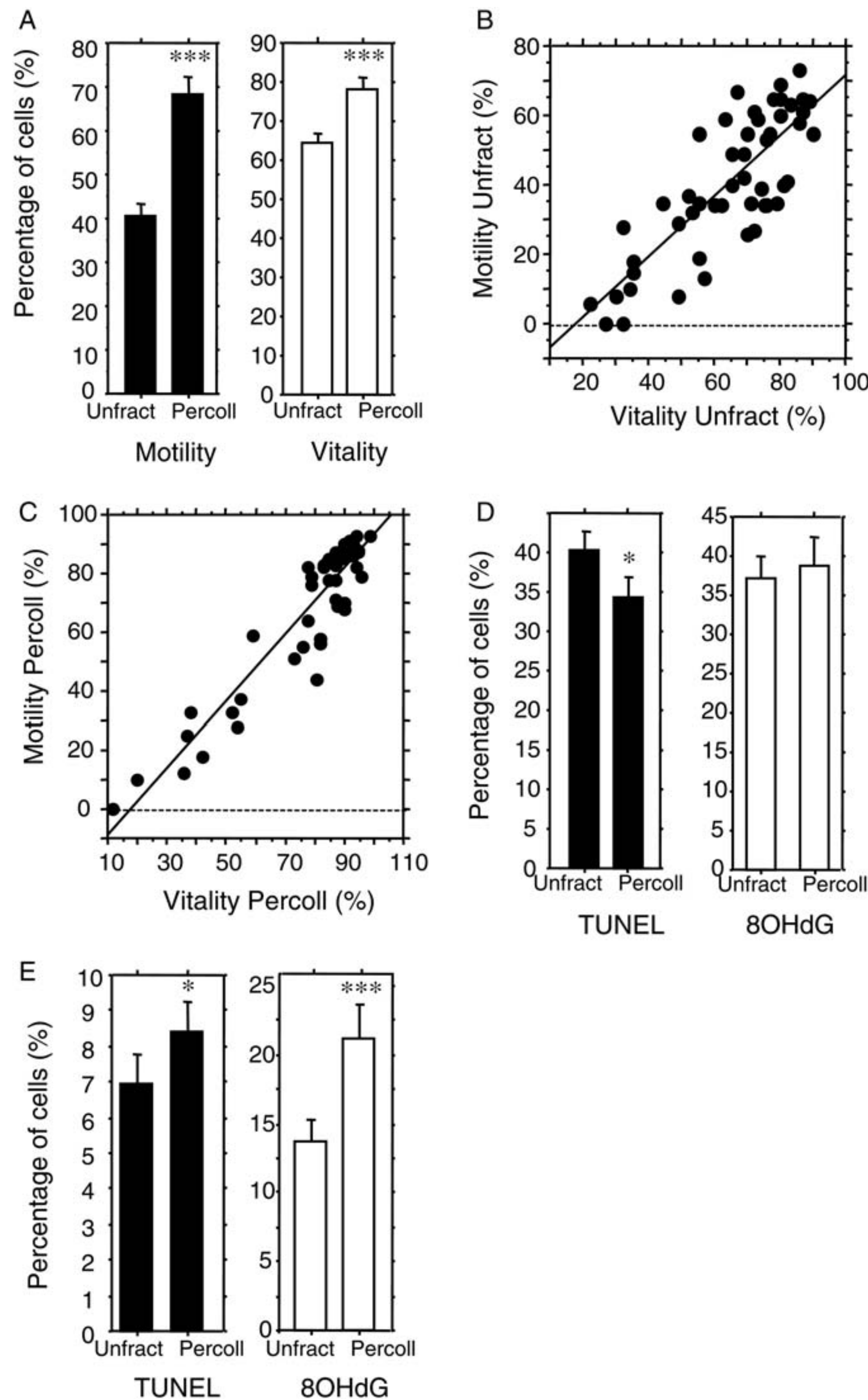

Figure 3 Impact of discontinuous density gradient centrifugation on sperm quality in the patient population ($n = 50$). (**A**) Percoll centrifugation resulted in a significant increase in both sperm motility (closed bars) and vitality (open bars) relative to unfractionated cells that had been centrifuged for exactly the same period of time in medium BWW. (**B**) The motility and vitality of the cells were highly correlated with the unfractionated sperm suspensions. (**C**) Motility and vitality were still highly correlated following Percoll gradient centrifugation, although the results for both parameters were shifted to a higher range of values. (**D**) The selection of motile, viable cells on Percoll gradients resulted in a significant decrease in the TUNEL activity (closed bars), although the 8OHdG values (open bars) remained unchanged. (**E**) Percoll centrifugation of patient samples resulted in significant increases in the percentage of spermatozoa that were alive and exhibiting either TUNEL or 8OHdG activity. *$P < 0.05$; ***$P < 0.001$.

Downloaded from https://academic.oup.com/humrep/article/25/10/2415/2385624 by guest on 19 April 2024

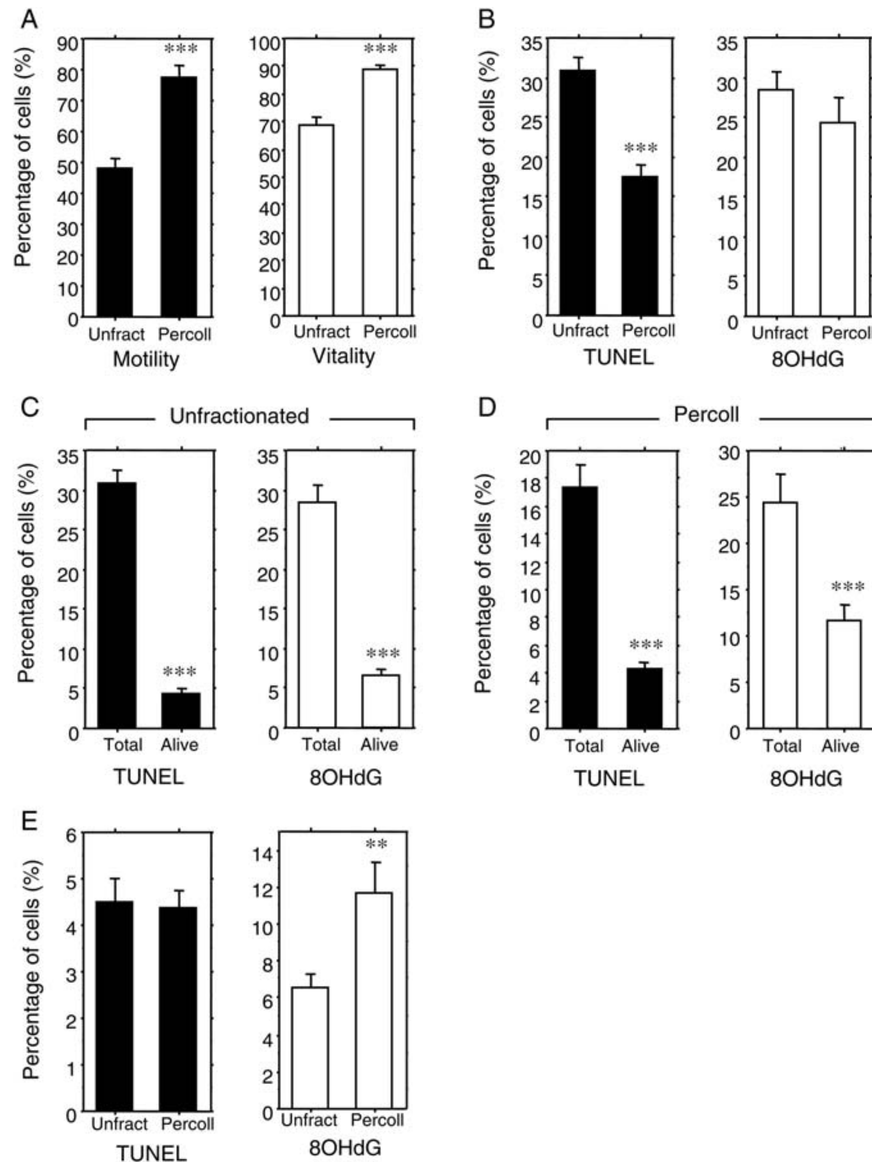

Figure 4 Impact of discontinuous density gradient centrifugation on TUNEL and 8OHdG activities in a cohort of unselected semen donors ($n = 36$). (**A**) Percoll centrifugation increased the proportion of motile (closed bars) and viable (open bars) cells in the sperm suspension. (**B**) When the total sperm population was considered Percoll fractionation significantly decreased the proportion of spermatozoa exhibiting a TUNEL signal (closed bar) while having no impact of 8OHdG expression (open bars), possibly because the selection of motile, viable cells is counteracted by the induction of oxidative stress as a consequence of Percoll fractionation. (**C**) Within the unfractionated sperm population, a majority of the TUNEL (closed bars) and 8OHdG (open bars) positive cells were non-viable. (**D**) Similarly, within the Percoll-prepared spermatozoa a majority of the TUNEL (closed bars) and 8OHdG (open bars) positive cells were non-viable. (**E**) Percoll centrifugation significantly increased the percentage of cells that were alive and exhibiting 8OHdG activity, while having no significant effect on the TUNEL signal. **$P < 0.01$; ***$P < 0.001$.

$P < 0.01$) and Percoll-prepared samples (donors, 17.4 ± 1.6% versus patients, 34.3 ± 2.5%; $P < 0.001$). Similarly the 8OHdG values were significantly different between these two populations for both the unfractionated (donors, 28.5 ± 2.1% versus patients, 37.3 ± 2.8%; $P < 0.05$) and Percoll prepared (donors, 24.3 ± 3.2% versus patients, 38.9 ± 3.2%; $P < 0.01$) sperm suspensions. These data were consistent with the notion that the patient and donor populations are distinctly different, with the former enriched with poor quality samples expressing high levels of DNA damage. In order to formally confirm this difference and develop objective thresholds for identifying abnormal samples, the frequency distribution of the data was examined.

Frequency distribution of the 8OHdG and TUNEL data

If either 8OHdG or TUNEL results are to be used as diagnostic criteria to select patients for therapeutic intervention, then two fundamental, practical questions have to be addressed: (i) should the

Downloaded from https://academic.oup.com/humrep/article/25/10/2415/2385624 by guest on 19 April 2024

measurements be made on unselected or Percoll prepared samples and (ii) what threshold values should be used for selection purposes? Both of these questions were addressed using ROC curve analysis. In conducting these analyses, we recognized that clear separation of the two groups (donors and patients) would not be achievable because the patient population would certainly include the fertile male partners of infertile women, while the donor population could feasibly include men suffering from some level of subfertility. Nevertheless, we did expect that the data distribution for the patient population to be significantly extended to the right, as proved to be the case.

An example of ROC curve analysis is given in Fig. 5; in this case, a plot of TUNEL positivity following Percoll fractionation. Such curves present a plot of sensitivity against 1 − specificity and provide a measure of the ability of a given test to distinguish between two populations (the donors and patients). The dashed diagonal represents completely overlapping distributions that cannot be separated; the farther the data points deviate from this no-discrimination line, the greater the diagnostic value of the test. The AUC gives a robust indication of the ability of a given test to discriminate two independent populations. In Table I, we present the AUC results for all of the treatments used in this study. It is clear from these data that the best diagnostic discrimination between patients and donors is achieved when 8OHdG or TUNEL activity is recorded for the entire sperm population not simply those that are alive, reflecting that fact that oxidative base adduct formation (8OHdG), DNA fragmentation and cell death lie on a continuum. For those tests focusing on the total cell population, it was also evident that Percoll fractionation gave the highest

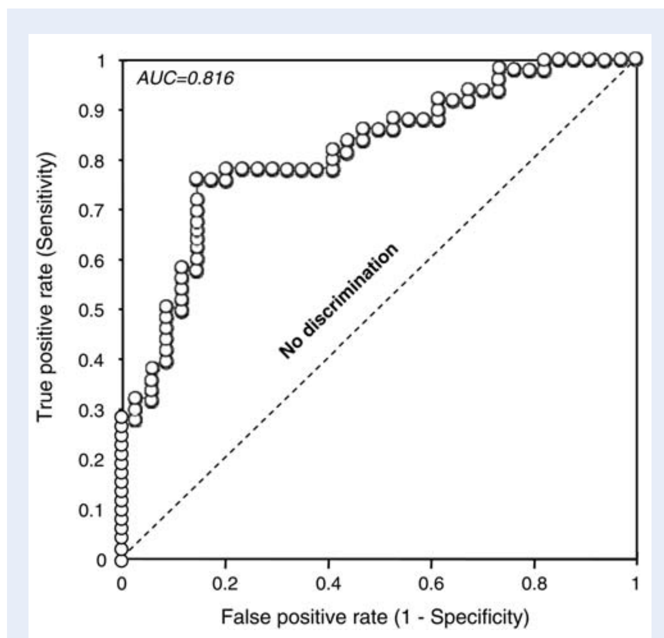

Figure 5 Example of an ROC curve. Dotted line represents the line of no discrimination. The further the data points are away from this diagonal, the more significant the criterion, as a means of discriminating the two populations under investigation. The relative strength of the discrimination can be deduced by calculating the AUC. In this example, the data represent the ROC curve describing TUNEL positivity following Percoll fractionation for discriminating between donor and patient samples.

and most significant AUC values (Table I). Using the Youden 'J' statistic as a guide to define optimized thresholds for discriminating between patients and donors, we could then go back to our data sets to determine how these measures performed as criteria for identifying men exhibiting abnormally high levels of DNA damage in their spermatozoa.

Analysis of the frequency distribution of the TUNEL data has allowed us, for the first time, to determine the DNA damage profile of patients and donors using a version of this assay that has been validated for use with human spermatozoa ((Mitchell *et al*., 2010). For the unfractionated spermatozoa, the frequency distribution of TUNEL-positive spermatozoa followed different trajectories in the patient and donor populations giving median (10th and 90th percentiles) values of 31.9% (18.1 and 42.7%) and 40.0% (20.0 $\pm$ 63.1%), respectively (Fig. 6A). Comparison of the frequency distributions for the unfractionated spermatozoa revealed that the patient data were extended to the right relative to the donors as anticipated (Fig. 6B and C). If a threshold of 40% positive cells was used as a selection criterion as suggested by the Youden index, then 48% of the patient cohort exceeded this value compared with 14% of the donor population. After Percoll centrifugation, the absolute TUNEL values were significantly reduced because the sperm populations had been enriched with viable cells. However, the difference between the donor and patient populations increased, generating median values of 30.9% (13.5 and 56.2%) and 15.9% (7.1 and 31.09%) for the patient and donor populations, respectively (Fig. 6D). The Youden threshold value of 24% (Table I) captured 70% of the patient population but only 15% of the donors, again emphasizing just how different these two populations are in terms of DNA damage.

Analysis of the frequency distribution data for 8OHdG again revealed a marked difference between the donor and patient cohorts in the distribution of results. In the donor population, the median 8OHdG value (10th and 90th percentiles) was 25.4% (14.8 and 43.3%) compared with 35.7% (15.7 and 62.1%) in the patient population (Fig. 7A). Using the Youden threshold of 40% for the unfractionated samples, 44% of the patient population were characterized as exhibiting abnormally high levels of oxidative DNA damage, as opposed to 14% of the controls (Fig. 7B and C). If the data distribution for 8OHdG after Percoll fractionation was considered, the difference between these two populations was even more marked. In this case, the median (10th and 90th percentiles) of the donor and patient populations were 17.1% (9.8 and 63.6%) and 30.2% (16.3 and 76.0%), respectively (Fig. 7D). After Percoll centrifugation, the distribution appeared more bimodal because the fractionation procedure had stimulated certain samples to generate high levels of 8OHdG activity. Because this induction of oxidative DNA damage affected both the donor and patient populations, discrimination between these groups becomes more difficult. Using the Youden threshold value of 24%, 29.3% of the donor population was classified as positive, compared with 68% of the patient population.

Discussion

The data generated in this study have provided the first insights into the size of the patient population exhibiting DNA fragmentation in their spermatozoa and the dependence of this condition on oxidative stress. It is clear from the data presented in Fig 2A that the incidence

Downloaded from https://academic.oup.com/humrep/article/25/10/2415/2385624 by guest on 19 April 2024

Table I ROC curve analysis for discriminating between the patients and donors.

Test	Fractionation	AUC	Standard error	Youden *J* (%)	Probability
Total TUNEL	Unfractionated	0.667	0.057	39.29	$P = 0.003$
Total TUNEL	Percoll	0.816	0.045	24.15	$P < 0.0001$
Total 8OHdG	Unfractionated	0.631	0.059	40.9	$P = 0.02$
Total 8OHdG	Percoll	0.745	0.055	23.47	$P < 0.0001$
Alive[1] TUNEL	Unfractionated	0.449	0.064	–	NS
Alive TUNEL	Percoll	0.659	0.059	4.5	$P = 0.007$
Alive 8OHdG	Unfractionated	0.572	0.064	–	NS
Alive 8OHdG	Percoll	0.612	0.062	–	NS

[1]Alive refers to the percentage of the entire sperm population that are alive and positive for the criterion under investigation.

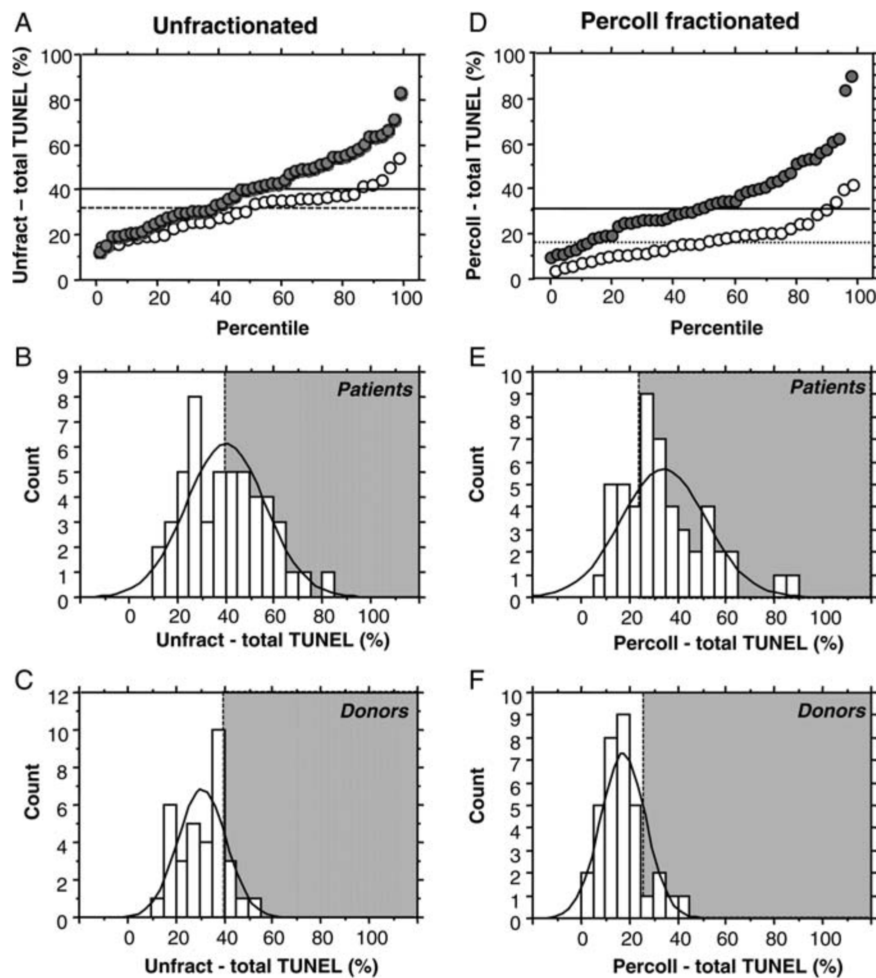

Figure 6 Frequency distribution analysis of the TUNEL data. (**A** and **D**) Frequency distribution of the TUNEL data for unfractionated and Percoll-fractionated sperm suspensions, respectively. Donor population plotted as open circles with 50th percentile represented by a dotted line; patient population plotted as closed circles with 50th percentile represented by a solid line. (**B**) Distribution of data for unfractionated patient samples; (**E**) distribution of data for Percoll-fractionated patient samples; (**C**) distribution of data for unfractionated donor samples; (**F**) distribution of data for Percoll-fractionated donor samples. For B, C, E and F, solid line represents calculated normal distribution; shaded area represents the samples that would be selected following the application of threshold values determined by Youdens's J. Unfract, unfractionated.

Downloaded from https://academic.oup.com/humrep/article/25/10/2415/2385624 by guest on 19 April 2024

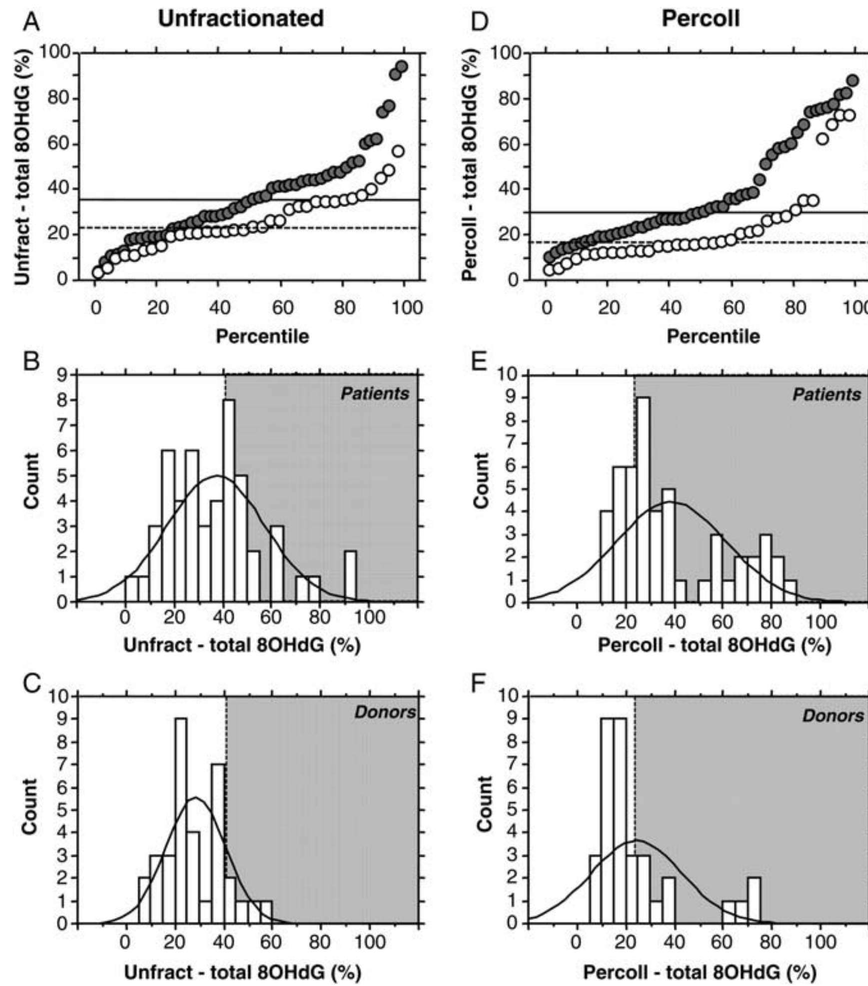

Figure 7 Frequency distribution analysis of the 8OHdG data. (**A** and **D**) Frequency distribution of the 8OHdG data for unfractionated and Percoll-fractionated sperm suspensions, respectively. Donor population plotted as open circles with 50th percentile represented by a dotted line; patient population plotted as closed circles with 50th percentile represented by a solid line. (**B**) Distribution of data for unfractionated patient samples; (**E**) distribution of data for Percoll-fractionated patient samples; (**C**) distribution of data for unfractionated donor samples; (**F**) distribution of data for Percoll-fractionated donor samples. For B, C, E and F solid line represents calculated normal distribution; shaded area represents the samples that would be selected following the application of threshold values determined by Youdens's J. Unfract, unfractionated.

of DNA fragmentation in viable spermatozoa recovered from assisted conception patients is highly correlated with the expression of 8OHdG, a marker for oxidative DNA damage. This finding reinforces the notion that a majority of the DNA damage seen in populations of human spermatozoa is associated with the onset of oxidative stress (De Iuliis *et al.*, 2009). However our data also indicate that a majority of the TUNEL- and 8OHdG-positive cells identified in this study are non-viable (Fig. 2B). Because the methodology we have used in this study uniquely allows the simultaneous assessment of vitality, DNA adduct formation and fragmentation, this is the first time that this relationship has been reported. Several important implications flow from this observation.

From a practical perspective, these results suggest that if a motile (and therefore live) cell is selected for ICSI (intra-cytoplasmic sperm injection), then there is a >90% chance that the cell will not possess any detectable DNA damage, since only ~5% of live cells are TUNEL positive after Percoll preparation (Fig. 2). The fact that sperm selection for ICSI conventionally focuses on the identification of motile, morphologically normal spermatozoa aligns perfectly with these conclusions (Van Steirteghem *et al.*, 1998; Nadalini *et al.*, 2009). The lack of a significant correlation between the incidence of DNA damage in sperm suspensions and the clinical outcomes of ICSI therapy, also emphasizes that the motile spermatozoon selected for injection, might not reflect the incidence of DNA damage in the population as a whole (Simon *et al.*, 2010). Conversely the positive correlations that have been observed between DNA damage in raw semen and the success of intrauterine insemination may depend on the ability of such DNA damage assays to reflect the underlying quality of the spermatogenic process rather than the fertilizing potential of individual cells (Bungum *et al.*, 2004).

In light of these findings, we might also expect density gradient centrifugation, a technique that is frequently used in assisted conception programmes to purify populations of viable motile cells (Aitken and Clarkson, 1988), to result in a reduction in the percentage of DNA

Downloaded from https://academic.oup.com/humrep/article/25/10/2415/2385624 by guest on 19 April 2024

damaged cells. In keeping with previous reports (Fariello et al., 2009), we also observed a reduction in TUNEL-positive cells when spermatozoa were prepared by discontinuous gradient centrifugation, presumably due to the positive selection of viable cells (Figs 3D and 4B). However, if we focused on just the viable spermatozoa, then discontinuous gradient centrifugation was found to increase the percentage of cells expressing high levels of 8OHdG resulting, in the patient population, in a significant increase in DNA fragmentation (Figs 3E and 4E).

This unexpected increase in oxidative DNA damage in Percoll-prepared spermatozoa presumably reflects the induction of free radical generation as the spermatozoa are centrifuged through a density gradient. Although we (Aitken and Clarkson, 1988), and others (McKinney et al., 1996), have previously reported that the act of centrifugation can stimulate ROS production by spermatozoa, this cannot explain the results observed in the present study because the unfractionated controls were subjected to exactly the same duration of centrifugation as the Percoll-prepared group. Rather, we propose that it is the shearing forces generated at the sperm surface as these cells are subjected to centrifugation through a dense gradient that triggers free radical generation and oxidative DNA damage. Physical perturbation of cell surfaces is known to create local membrane depolarization and ROS generation by NADPH oxidases and NO synthases (Amatore et al., 2008). Whether such enzyme systems are involved in triggering oxidative stress in spermatozoa subjected to discontinuous gradient centrifugation will be examined in future studies. It would also be interesting to know whether the oxidative stress created by density gradient centrifugation has long-term impacts on the motility, vitality and levels of DNA damage sustained by the spermatozoa as a function of the time elapsed since preparation. Whatever the mechanism, such iatrogenically induced oxidative DNA damage was observed in both patient and donor samples (Figs 3E and 4E), although only in the former did such lesions lead to an increase in DNA fragmentation. Such differences are presumably quantitative rather than qualitative and reflect the generally higher levels of oxidative stress seen in the patient samples. Our current thinking is that free radical generation, oxidative DNA damage, DNA fragmentation and cell death lie on a continuum and that spermatozoa from the patient population are more advanced along this path than those from the donors.

If these conclusions are correct, and oxidative stress is at the heart of the increased DNA damage we see in the patient population, then antioxidant therapy would seem a logical means of addressing this problem. The notion that antioxidants are important for male reproduction has been around since the 1930s when vitamin E was shown to be an essential requirement for normal testicular function (Mason et al., 1930). A possible role for antioxidants in the treatment of male infertility was also supported by more recent prospective studies demonstrating that free radical generation by human spermatozoa is predictive of fertility in couples possessing a normal female partner (Aitken et al., 1991). A small number of studies have been performed with the aim of assessing the possible therapeutic value of antioxidants in the treatment of male infertility (Tremellen, 2008). However, none have delivered a definitive result because they have been underpowered, have lacked appropriate controls, have not incorporated a double-blind crossover design or have not selected an appropriate group of patients. If antioxidants are to be used therapeutically, a rational cohort for treatment would be that subpopulation of males exhibiting signs of oxidative damage to their sperm DNA. Surprisingly, none of the previous investigations of antioxidant therapy for male infertility have used oxidative DNA damage as a selection criterion. The formation of 8OHdG constitutes a robust marker for oxidative DNA damage (De Iuliis et al., 2009), however before it can be used as a selection criterion for antioxidant therapy, it is important to determine: which population of spermatozoa will be assessed, how it will be prepared and what threshold values will be used to select patients for treatment?

In terms of the sperm subpopulation to be assessed, we have the choice of selecting either the total proportion of 8OHdG-positive cells or just selecting those that are alive and 8OHdG positive. In this context, we would argue that the measurement should be made on the entire sperm population because a loss of viability is part of a progressive oxidative process that begins with the excessive generation of ROS, leads to lipid peroxidation and oxidative DNA damage, and culminates in DNA fragmentation and death. Whether oxidatively damaged cells are alive or dead is just a question of the time that has elapsed between the initiation of oxidative stress and the point of assessment.

In terms of sperm preparation, we could either measure 8OHdG in unfractionated sperm populations or those prepared by discontinuous gradient density centrifugation. The latter increases the proportion of viable cells but also simultaneously increases the proportion of viable cells exhibiting 8OHdG formation due to the artificial creation of oxidative stress (Fig. 3E and 4E). It could be argued that such stimulation accentuates the difference between normal and abnormal cells (Fig. 7) and so should be used to prepare the cells for diagnostic purposes. However, the artificial nature of this stimulation argues against its use in a diagnostic context, even though this side effect of discontinuous density gradient centrifugation is clearly important therapeutically. On balance, we would suggest that 8OHdG measurements should be made on the total population of unfractionated cells, to give an overall view of oxidative stress in the ejaculate.

DNA damage, whether measured as fragmentation or oxidized base adduct formation, follows a normal distribution in unfractionated sperm suspensions (Figs 6 and 7). In order to find a threshold within this distribution for identifying men with abnormally high levels of DNA damage, ROC curve analysis in combination with Youden's *J* statistic was used. With this approach we could compare the distribution of data in a patient population attending an IVF programme, which should contain a subpopulation of infertile males, with a donor population, in which subfertility would be relatively scarce. Accordingly, the frequency distribution of data for both the TUNEL and 8OHdG assays was found to be extended to the right in the patient population (Figs 6 and 7). The results of this analysis were also consistent in recommending a diagnostic threshold of around 40% positive cells for both TUNEL and 8OHdG formation in unfractionated sperm suspensions (Figs 6 and 7). Using this threshold, subpopulations of spermatozoa suffering from oxidative DNA damage can now be readily identified, as a prelude to antioxidant therapy.

Authors' roles

R.J.A. contributed to the study design, analysed and interpreted the data and drafted the paper; G.D. participated in the study design and preparation of the manuscript; J.F. performed the analyses; A.H.

Downloaded from https://academic.oup.com/humrep/article/25/10/2415/2385624 by guest on 19 April 2024

undertook the clinical evaluation of the patients and R.M. contributed to the study design and manuscript preparation.

Acknowledgements

The authors gratefully acknowledge the contribution of Jodi Powell who orchestrated the semen donor panel.

Funding

The authors acknowledge financial support from the NHMRC (program grant #494802) and the Hunter Medical Research Institute (HMRI).

References

Aitken RJ. The role of free radicals and sperm function. *Int J Androl* 1989; **12**:95–97.

Aitken RJ. The Amoroso Lecture. The human spermatozoon—a cell in crisis? *J Reprod Fert* 1999;**115**:1–7.

Aitken RJ, Clarkson JS. Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. *J Reprod Fert* 1987;**81**:459–469.

Aitken RJ, Clarkson JS. Significance of reactive oxygen species and antioxidants in defining the efficacy of sperm preparation techniques. *J Androl* 1988;**9**:367–376.

Aitken RJ, Clarkson JS, Fishel S. Generation of reactive oxygen species, lipid peroxidation and human sperm function. *Biol Reprod* 1989; **41**:183–187.

Aitken RJ, Irvine DS, Wu FC. Prospective analysis of sperm-oocyte fusion and reactive oxygen species generation as criteria for the diagnosis of infertility. *Am J Obstet Gynecol* 1991;**164**:542–551.

Aitken RJ, Gordon E, Harkiss D, Twigg JP, Milne P, Jennings Z, Irvine DS. Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. *Biol Reprod* 1998; **59**:1037–1046.

Aitken RJ, De Iuliis GN, McLachlan RI. Biological and clinical significance of DNA damage in the male germ line. *Int J Androl* 2009;**32**:46–56.

Alvarez JG, Touchstone JC, Blasco L, Storey BT. Spontaneous lipid peroxidation and production of hydrogen peroxide and superoxide in human spermatozoa. *J Androl* 1987;**8**:338–348.

Amatore C, Arbault S, Erard M. Triangulation mapping of oxidative bursts released by single fibroblasts by amperometry at microelectrodes. *Anal Chem* 2008;**80**:9635–9641.

Barratt CL, Aitken RJ, Björndahl L, Carrell DT, de Boer P, Kvist U, Lewis SE, Perreault SD, Perry MJ, Ramos L *et al*. Sperm DNA: organization, protection and vulnerability: from basic science to clinical applications–a position report. *Hum Reprod* 2010;**25**:824–838.

Biggers JD, Whitten WK, Whittingham DG. The culture of mouse embryos in vitro. In: JC Daniel (ed). *Methods in Mammalian Embryology*. San Francisco: Freeman Press, 1971,86–94. USA.

Bungum M, Humaidan P, Spanò M, Jepson K, Bungum L, Giwercman A. The predictive value of sperm chromatin structure assay (SCSA) parameters for the outcome of intrauterine insemination, IVF and ICSI. *Hum Reprod* 2004;**19**:1401–1408.

Carrell DT, Liu L, Peterson CM, Jones KP, Hatasaka HH, Erickson L, Campbell B. Sperm DNA fragmentation is increased in couples with unexplained recurrent pregnancy loss. *Arch Androl* 2003;**49**:49–55.

De Iuliis GN, Thomson LK, Mitchell LA, Finnie JM, Koppers AJ, Hedges A, Nixon B, Aitken RJ. DNA damage in human spermatozoa is highly correlated with the efficiency of chromatin remodeling and the formation of 8-hydroxy-2'-deoxyguanosine, a marker of oxidative stress. *Biol Reprod* 2009;**81**:517–524.

Fariello RM, Del Giudice PT, Spaine DM, Fraietta R, Bertolla RP, Cedenho AP. Effect of leukocytospermia and processing by discontinuous density gradient on sperm nuclear DNA fragmentation and mitochondrial activity. *J Assist Reprod Genet* 2009;**26**:151–157.

Greco E, Iacobelli M, Rienzi L, Ubaldi F, Ferrero S, Tesarik J. Reduction of the incidence of sperm DNA fragmentation by oral antioxidant treatment. *J Androl* 2005;**26**:349–353.

Jones R, Mann T, Sherins R. Peroxidative breakdown of phospholipids in human spermatozoa, spermicidal properties of fatty acid peroxides, and protective action of seminal plasma. *Fertil Steril* 1979; **31**:531–537.

Kessopoulou E, Powers HJ, Sharma KK, Pearson MJ, Russell JM, Cooke ID, Barratt CL. A double-blind randomized placebo cross-over controlled trial using the antioxidant vitamin E to treat reactive oxygen species associated male infertility. *Fertil Steril* 1995;**64**:825–831.

Kodama H, Yamaguchi R, Fukuda J, Kasi H, Tanak T. Increased deoxyribonucleic acid damage in the spermatozoa of infertile male patients. *Fertil Steril* 1997;**65**:519–524.

Koppers AJ, De Iuliis GN, Finnie JM, McLaughlin EA, Aitken RJ. Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa. *J Clin Endocrinol Metab* 2008;**93**:3199–3207.

Koppers AJ, Garg ML, Aitken RJ. Stimulation of mitochondrial reactive oxygen species production by unesterified, unsaturated fatty acids in defective human spermatozoa. *Free Radic Biol Med* 2010; **48**:112–119.

Lewis SE, Aitken RJ. DNA damage to spermatozoa has impacts on fertilization and pregnancy. *Cell Tissue Res* 2005;**322**:33–41.

Mason KE. The specificity of vitamin E for the testis. I. Relation between vitamins A and E. *J Exp Zool* 1930;**55**:101–122.

McKinney KA, Lewis SE, Thompson W. Reactive oxygen species generation in human sperm: luminol and lucigenin chemiluminescence probes. *Arch Androl* 1996;**36**:119–125.

Meseguer M, Martínez-Conejero JA, O'Connor JE, Pellicer A, Remohí J, Garrido N. The significance of sperm DNA oxidation in embryo development and reproductive outcome in an oocyte donation program: a new model to study a male infertility prognostic factor. *Fertil Steril* 2008;**89**:1191–1199.

Mitchell LA, De Iuliis GN, Aitken RJ. The TUNEL assay consistently underestimates DNA damage in human spermatozoa and is influenced by DNA compaction and cell vitality: development of an improved methodology. *Int J Androl* 2010 (in press).

Morris ID, Ilott S, Dixon L, Brison DR. The spectrum of DNA damage in human sperm assessed by single cell gel electrophoresis (comet assay) and its relationship to fertilization and embryo development. *Hum Reprod* 2002;**17**:990–998.

Nadalini M, Tarozzi N, Distratis V, Scaravelli G, Borini A. Impact of intracytoplasmic morphologically selected sperm injection on assisted reproduction outcome: a review. *Reprod Biomed Online* 2009; **19**(Suppl. 3):45–55.

Seli E, Gardner DK, Schoolcraft WB, Moffatt O, Sakkas D. Extent of nuclear DNA damage in ejaculated spermatozoa impacts on blastocyst development after *in vitro* fertilization. *Fertil Steril* 2004; **82**:378–383.

Simon L, Brunborg G, Stevenson M, Lutton D, McManus J, Lewis SE. Clinical significance of sperm DNA damage in assisted reproduction outcome. *Hum Reprod* 2010 (in press).

Suleiman SA, Elamin Ali M, Zaki ZMS, El-Malik EMA, Nasr MA. Lipid peroxidation and human sperm motility: protective role of vitamin E. *J Androl* 1996;**17**:530–537.

Downloaded from https://academic.oup.com/humrep/article/25/10/2415/2385624 by guest on 19 April 2024

Tremellen K. Oxidative stress and male infertility—a clinical perspective. *Hum Reprod Update* 2008;**14**:243–258.

Van Steirteghem A, Nagy P, Joris H, Janssenswillen C, Staessen C, Verheyen G, Camus M, Tournaye H, Devroey P. Results of intracytoplasmic sperm injection with ejaculated, fresh and frozen-thawed epididymal and testicular spermatozoa. *Hum Reprod* 1998;**13**(Suppl. 1):134–142.

Virro MR, Larson-Cook KL, Evenson DP. Sperm chromatin structure assay parameters are related to fertilization, blastocyst development, and ongoing pregnancy in *in vitro* fertilization and intracytoplasmic sperm injection cycles. *Fertil Steril* 2004;**81**: 1289–1295.

Zini A, Sigman M. Are tests of sperm DNA damage clinically useful? Pros and cons. *J Androl* 2009;**30**:219–229.

Downloaded from https://academic.oup.com/humrep/article/25/10/2415/2385624 by guest on 19 April 2024