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Potential importance of transition metals in the induction of DNA damage by sperm preparation media

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STUDY QUESTION: What are the mechanisms by which the preparation of spermatozoa on discontinuous density gradients leads to an increase in oxidative DNA damage?

SUMMARY ANSWER: The colloidal silicon solutions that are commonly used to prepare human spermatozoa for assisted reproduction technology (ART) purposes contain metals in concentrations that promote free radical-mediated DNA damage.

WHAT IS KNOWN ALREADY: Sporadic reports have already appeared indicating that the use of colloidal silicon-based discontinuous density gradients for sperm preparation is occasionally associated with the induction of oxidative DNA damage. The cause of this damage is however unknown.

STUDY DESIGN, SIZE, DURATION: This study comprised a series of experiments designed to: (i) confirm the induction of oxidative DNA damage in spermatozoa prepared on commercially available colloidal silicon gradients, (ii) compare the levels of damage observed with alterative sperm preparation techniques including an electrophoretic approach and (iii) determine the cause of the oxidative DNA damage and develop strategies for its prevention. The semen samples employed for this analysis involved a cohort of >50 unselected donors and at least three independent samples were used for each component of the analysis.

PARTICIPANTS/MATERIALS, SETTING, METHODS: The setting was a University biomedical science laboratory. The major techniques employed were: (i) flow cytometry to study reactive oxygen species generation, lipid peroxidation and DNA damage, (ii) computer-aided sperm analysis to measure sperm movement and (iii) inductively coupled mass spectrometry to determine the elemental composition of sperm preparation media.

MAIN RESULTS AND THE ROLE OF CHANCE: Oxidative DNA damage is induced in spermatozoa prepared on PureSperm® discontinuous colloidal silicon gradients ($P < 0.001$ versus repeated centrifugation) because this medium contains metals, particularly Fe, Al and Cu, which are known to promote free radical generation in the immediate vicinity of DNA. This damage can be significantly accentuated by reducing agents, such as ascorbate ($P < 0.001$) and inhibited by selective chelation ($P < 0.001$). This problem is not confined to PureSperm®; analysis of additional commercial sperm preparation media revealed that metal contamination is a relatively constant feature of such products.

LIMITATIONS, REASONS FOR CAUTION: While the presence of metals, particularly transition metals, may exacerbate the levels of oxidative DNA damage seen in human spermatozoa, the significance of such damage has not yet been tested in suitably powered clinical trials.

WIDER IMPLICATIONS OF THE FINDINGS: The results explain why the preparation of spermatozoa on discontinuous colloidal silicon gradients can result in oxidative DNA damage. The results are of immediate relevance to the development of safe, effective protocols for the preparation of spermatozoa for ART purposes.

STUDY FUNDING/COMPETING INTEREST(S): The study was funded by the Australian Health and Medical Research Council. One of the authors (R.J.A.) has had a consultantship with a biotechnology company, NuSep, interested in the development of electrophoretic methods of sperm preparation. He has no current financial interest in this area. None of the other authors have a conflict of interest to declare.

Key words: sperm DNA damage / colloidal silicon / sperm preparation / assisted reproduction technology / transition metals

Introduction

DNA damage in human spermatozoa is a relatively common condition that has been linked with a variety of adverse clinical outcomes including impaired fertilization, disrupted preimplantation development, an increased incidence of miscarriage, reduced live birth rate and morbidity in the offspring, including childhood cancer (Aitken *et al.*, 2009, 2012a; Barratt *et al.*, 2010; Simon *et al.*, 2013). The major cause of DNA damage appears to be oxidative stress coupled with poor compaction of sperm chromatin (De Iuliis *et al.*, 2009) and a marked tendency of human spermatozoa to default to an apoptotic pathway dominated by the mitochondrial generation of reactive oxygen species (ROS) (Koppers *et al.*, 2011). Once ROS generation in spermatozoa has been initiated it tends to become a self-perpetuating redox cycle during which electrophilic cytotoxic aldehydes, such as 4-hydroxynonenal (4HNE), generated as a result of ROS-induced lipid peroxidation, bind to the nucleophilic centres of sperm proteins involved in the mitochondrial electron transport chain. This adduction process disrupts the efficient movement of electrons along the mitochondrial electron transport chain and enhances ROS generation, stimulating lipid peroxidation and the generation of yet more cytotoxic aldehydes that perpetuate the cycle of ROS induced, ROS generation culminating in severe oxidative stress, apoptosis and death (Aitken *et al.*, 2012b).

Oxidative stress in the male germ line results in the generation of oxidized DNA base adducts such as 8-hydroxy-2′-deoxyguanosine (8OHdG), which are readily detected in the spermatozoa of infertile men (Kodama *et al.*, 1997; Aitken *et al.*, 2010). Spermatozoa are vulnerable to such attack because they only possess the first enzyme in the base excision repair pathway, 8-oxoguanine glycosylase 1 (Smith *et al.*, 2013). *In vivo* situations associated with 8OHdG formation in spermatozoa include the oxidative stress associated with heavy smoking, low levels of antioxidant protection, a sedentary lifestyle, male infertility associated with varicocele formation, exposure to cadmium and polymorphisms in key antioxidant genes including glutathione S-transferase, PON1 and SOD2 (Fraga *et al.*, 1996; Shen *et al.*, 1999; Chen *et al.*, 2002; Xu *et al.*, 2003; Chigurupati *et al.*, 2008; Sakamoto *et al.*, 2008; Aitken *et al.*, 2010; Ji *et al.*, 2012; Tang *et al.*, 2012). *In vitro*, iatrogenic induction of 8OHdG formation in spermatozoa is also possible as a consequence of both cryostorage (Thomson *et al.*, 2009) and the use of discontinuous density gradients to prepare the spermatozoa for IVF (Aitken *et al.*, 2010). The latter finding (Aitken *et al.*, 2010) was unexpected because discontinuous gradient centrifugation is well known to generate sperm populations with enhanced motility and morphology as well as high purity (Pousette *et al.*, 1986; Aitken and Clarkson, 1988; Claassens *et al.*, 1996). Nevertheless, high levels of DNA damage have been observed by others who have undertaken the preparation of spermatozoa by discontinuous density gradient centrifugation using either Percoll® (Zini *et al.*, 1999, 2000) or ISolate® (Stevanato *et al.*, 2008). The purpose of this study was to examine the levels of oxidative DNA damage observed when a density gradient medium designed for use in routine clinical practice (PureSperm®) is used for sperm isolation purposes and to determine the underlying mechanisms responsible for the changes observed.

Materials and Methods

Sperm preparation

This research was based on a cohort of unselected healthy donors to our reproductive research programme, a majority of whom were normozoospermic students of unknown fertility status (Supplementary data, Table SI); a total of 64 donors were used in the course of this research with an average (± SEM) of 1.4 ± 0.1 ejaculates per donor. Scientific use of these samples for research purposes was approved by our Institutional Human Ethics Committee and the State Minister for Health and informed written consent for use of these samples was obtained from all donors. 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. Purification of human spermatozoa was achieved using cycles of repeated centrifugation and resuspension (500*g* for 5 min) in medium Biggers, Whitten and Whittingham (BWW) containing 1 mg/ml polyvinyl alcohol (PVA) instead of bovine serum albumin (Biggers *et al.*, 1971) or centrifugation on discontinuous gradients created using 45 and 90% PureSperm® (silane-coated colloidal silicon particles from Nidacon, Gothenberg, Sweden) as previously described for Percoll (Mitchell *et al.*, 2011) After 30 min centrifugation at 500*g*, purified spermatozoa were recovered from the base of the discontinuous gradients, washed with BWW and finally resuspended at a concentration of 6×10^6 cell/ml in BWW. In order to ensure that minor variations in preparation protocol did not fundamentally impact upon the results secured in this study, supplementary studies were also performed in which the centrifugation time (20 min), *g*-force (300*g*) and gradient composition (40 and 80%) were reduced to levels that are commonly employed in a clinical setting and recommended by the manufacturer (Nidacon). In this case the levels of DNA damage were compared with swim-up spermatozoa prepared by gently overlayering 1 ml semen with 1.5 ml of BWW in a 15 ml conical-bottomed Falcon tube, which was inclined at 45° and incubated for 60 min at 37°C. The uppermost layer, containing the most motile spermatozoa was then removed and the spermatozoa suspended in medium BWW for analysis. In a separate suite of experiments, spermatozoa were prepared by electrophoresis (14 V for 5 min) using the CS10 electrophoretic sperm isolation system as described by Aitken *et al.* (2011). Motility was determined using phase contrast optics, while vitality was assessed using the eosin exclusion test (Aitken *et al.*, 2010). For all comparative studies these different sperm isolation techniques were applied to semen samples that were split immediately prior to preparation.

Flow cytometry assays

MitoSOX Red™ (MSR) (Molecular Probes, OR, USA) and dihydroethidium (DHE) (Molecular Probes) for mitochondrial and general cellular superoxide generation, respectively, were performed by flow cytometry incorporating SYTOX G green as a vitality stain, as described previously (Koppers *et al.*, 2008, 2011); 2.0×10^6 spermatozoa were used per sample and these probes were incubated with the spermatozoa for 15 min at 37°C. Non-sperm-specific events were gated out and 10 000 cells were examined. The results were expressed as the percentage of viable cells staining positively with each probe.

The presence of the oxidized DNA base adduct, 8OHdG, was also assessed by flow cytometry using the OxyDNA Assay (Calbiochem, La Jolla, CA, USA) in combination with LIVE/DEAD® fixable far red, dead cell stain (Molecular Probes) as described by Koppers *et al.* (2011). As a

measure of lipid peroxidation, the small molecular mass electrophile, 4HNE, was also monitored by flow cytometry (Aitken *et al.*, 2012b).

All flow cytometry analyses reported in this study were conducted on a fluorescence-activated cell sorter (FACS)-Calibur or an FACS-Canto flow cytometer (Becton Dickinson, Mountain View, CA, USA) with a 488 nm argon laser. Forward scatter and side scatter measurements were taken to generate a scatter plot, which was used to gate for sperm cells only, excluding any larger contaminating cells. All data were acquired and analysed using CellQuest Pro (FACS-Calibur) or BD Diva (FACS-Canto) software (Becton Dickinson) and a total of 10 000 events were collected per sample.

Chemiluminescence

For lucigenin-dependent chemiluminescence, 4×10^6 spermatozoa in 400 μl BWW were supplemented with 4 μl lucigenin (25 mM) and the samples were then run for 5 min at 37°C in a Berthold AutoLumat luminometer LB-953 (Berthold, Bad Wildbad, Germany) to stabilize the chemiluminescent system. Chemiluminescence was ultimately monitored for 40 min and the results expressed as integrated counts. The luminol-peroxidase assay was performed on 5×10^6 spermatozoa in 400 μl BWW and was essentially the same as the lucigenin assay except that the cells were supplemented with 4 μl luminol (25 mM) and 8 μl horse-radish peroxidase (HRP, 11.52 U/ml). For both chemiluminescence assays, media blanks were run for every treatment in order to ensure that the signals recorded were not due to the spontaneous activation of the probe.

DNA damage assays

The levels of DNA damage were assessed by the modified terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay as described by Mitchell *et al.* (2011) involving a nuclear decondensation step with dithiothreitol prior to the assessment of DNA strand breaks. In addition, sperm chromatin status was assessed with the sperm chromatin structure assay (SCSA). For this procedure, spermatozoa were washed with BWW, snap frozen in liquid nitrogen and stored at −80°C. SCSA was performed as described by Evenson and Jost (2000) using an FACScan Flow Cytometer with CellQuest software (Becton Dickinson, San Diego, CA, USA). Briefly, 200 μl acid detergent solution (0.08 N HCl, 0.1 5 M NaCl, 0.1% Triton X-100, pH 1.2) was added to thawed cells. Following a 30 s incubation, 600 μl acridine orange staining solution was added and the sample was run through the flow cytometer for 2.5 min before acquisition. A total of 5000 events were recorded following gating out of debris. Analysis was performed using SCSAsoft Version 1.0 software (SCSA Diagnostics, Volga, SD, USA) and the DNA fragmentation index calculated.

Chromomycin 3

The efficiency of sperm chromatin protamination was determined using chromomycin 3 (CMA3), a fluorescent probe that competes with protamines for binding sites on the DNA such that the lower the level of protamination, the greater the degree of sperm fluorescence (Bianchi *et al.*, 1993; De Iuliis *et al.*, 2009). Briefly, spermatozoa were fixed with 4% paraformaldehyde for 15 min at 4°C, washed for 5 min and stored at 4°C in 0.1 M glycine for a maximum of 1 week at 4°C. An aliquot of the sperm suspension was subsequently settled onto a poly-L-lysine coverslip in a humidified chamber for at least 2 h at 4°C. The spermatozoa were then permeabilized in 0.2% Triton X-100 in phosphate-buffered saline for 15 min at 37°C and washed once in McIlvaine's buffer. Coverslips were then stained with 25 μl of CMA3 solution (0.25 mg/ml in McIlvaine's buffer) at room temperature for 20 min, protected from light. The cells were subsequently rinsed 3 × in McIlvaine's buffer and mounted in 10% mowiol 4-88 (Calbiochem) with 30% glycerol in 0.2 M Tris (pH 8.5) containing 2.5% 1,4-diazobicyclo-[2.2.2]-octane (DABCO). Cells ($n = 100$) were examined using a Zeiss Axioplan 2 fluorescence microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA).

Sperm movement characteristics

The movement characteristics of the spermatozoa were assessed using a Hamilton-Thorn motility analyzer (HTMA IVOS; Hamilton-Thorn Research, Danvers, MA, USA). The settings for human spermatozoa were as follows: negative phase-contrast optics, recording rate 60 frames/s, minimum contrast 80, minimum cell size 3 pixels, low size gate 1.0, high size gate 2.9, low intensity gate 0.6, high intensity gate 1.4, non-motile head size 6 and non-motile head intensity 160. The criteria of sperm movement assessed were curvilinear velocity (VCL, the two-dimensional track described by the sperm head in unit time); average path velocity (VAP, the average path described by each spermatozoon in unit time, calculated using a 5-point smoothing algorithm); straight line velocity (VSL, the straight line distance travelled by the sperm head in unit time); the progressive VAP threshold value was 25 μm/s, the slow cells VAP cut off, 5 μm/s; the slow cell VSL (straight line velocity) cut off, 11 μm/s and the threshold STR (straightness) >80%. Progressive cells were those exhibiting a VAP of >25 μm/s and an STR of >80%, while percentage rapid alluded to the proportion of spermatozoa exhibiting a VAP of >25 μm/s. Linearity (LIN) was defined as the VSL/VCL × 100, while STR was VSL/VAP × 100.

Inductively coupled plasma-mass spectrometry

The quadrupole inductively coupled plasma-mass spectrometry (ICP-MS) instrument used was the Agilent 7700x (Agilent Technologies, Santa Clara, USA) equipped with an integrated autosampler and third-generation Octopole reaction system. Data acquisition and post-analysis was performed using G7201A ICP-MS MassHunter Workstation Software, Rev. A.01.02 (Agilent Technologies). The instrument was operated in both 'No Gas' and 'He' tunes modes.

All samples were provided to the laboratory in polypropylene tubes and kept refrigerated until ready for initial sample preparation. A 0.5 ml aliquot of each sample was then transferred to a certified metal-free, 15 ml polypropylene tube (Metal Free™, Labcon North America, USA) to which 0.2 ml of ultra-high-purity nitric acid was added (BASELINE®, Seastar Chemicals, Canada). All samples were then diluted to a final volume of 10 ml (1:20 dilution) with ICP-MS-grade ultrapure water (High Purity Standards, USA). Samples were then homogenized and allowed to digest, *in situ*, for 24 h before analysis. A background (matrix) blank was also prepared by diluting 0.2 ml of ultra-high-purity nitric acid to a final volume of 10 ml with ICP-MS-grade ultrapure water in a certified metal-free polypropylene tube. All samples for analysis, except the background blank, were spiked to 100 ppb (100 μg/l) with both internal standards, Y and Rh (Merck Chemicals, Germany). Calibration standards were prepared from a commercially available ICP multi-elemental standard (Merck Chemicals, Germany, Cat. # 110580), containing 30 elements. All calibration standards, including the calibration blank, were diluted with or prepared from a commercially available, ICP-MS-grade, 2% nitric acid solution (High Purity Standards) and spiked to 100 ppb (100 μg/l) with both internal standards. Detection limits for all measured elements ranged from <1 to <0.1 ppb. The data analysis was handled by the Data Analysis module of the ICP-MS control software. Sample dilutions were automatically corrected for by the software and all reported concentrations are expressed as mg/l. Calibration plots for all measured elements were linear with all correlation coefficients $r = >0.999$. Method and instrument validation was accomplished using quality control check samples, which were analysed after all samples were processed. The reported values for all analytes correlated well with their theoretical values.

Statistical analysis

All experiments were replicated at least three times on independent samples and the results analysed by one- and two- way ANOVA using the SuperANOVA program (Abacus Concepts, Inc., CA, USA) on a MacIntosh G4 Powerbook computer; *post hoc* comparison of group means was by Fisher's protected least significant difference (PLSD). Paired comparisons were conducted using a paired *t*-test using the Statview program (Abacus Concepts, Inc.). Differences with a *P* value of $<0.05\%$ were regarded as significant. All data are presented as the mean value $\pm$ SEM.

Results

Impact of discontinuous gradient centrifugation and 8OHdG formation

An initial analysis of total 8OHdG in 12 independent samples within 30 min of being prepared by repeated centrifugation (25.8 $\pm$ 1.4%) or PureSperm® (48.2 $\pm$ 5.9%) demonstrated that oxidative DNA damage was significantly elevated in the latter ($P < 0.01$). In light of these data, this comparison between repeated centrifugation and PureSperm® was reiterated and additional measurements made of other sperm parameters, with particular reference to oxidative stress. Again, the spermatozoa were prepared as described in Materials and Methods, resuspended in medium BWW and subjected to a range of analyses (8OHdG, CMA3, motility, mitochondrial ROS and total cellular ROS) within 30 min of preparation. In this study a highly significant ($P < 0.001$) increase in 8OHdG formation was once more observed (Fig. 1A); however, this damage was not associated with any impairment in the ability of PureSperm® to select high-quality cells since the incidence of poor protamination (CMA3 fluorescence; Fig. 1B) was reduced, while motility was enhanced (Fig. 1C) following the use of this preparation medium. The high levels of 8OHdG formation were also not related to ROS generation because the output of mitochondrial ROS (Fig. 1D) was unchanged, while total cellular ROS production was significantly reduced ($P < 0.05$; Fig. 1E). Furthermore, chemiluminescence measurements, which largely reflect levels of leucocyte contamination (Aitken *et al.*, 2013), demonstrated that the unfractionated samples were much more redox active than the PureSperm®-prepared sperm suspensions, whether the probe employed was luminol/peroxidase (Supplementary data, Fig. S1A) or lucigenin (Supplementary data, Fig. S1B). The lack of an association between the induction of oxidative DNA damage by PureSperm® and generalized oxidative stress was also emphasized by measurements of 4HNE, an electrophilic end-product of lipid peroxidation. While 8OHdG was consistently elevated in these samples (Supplementary data, Fig. S1C), levels of 4HNE production were no different from unselected samples prepared by repeated centrifugation (Supplementary data, Fig. S1D).

Comparison of sperm isolation techniques

In order to ensure that this increase in oxidative DNA damage was not a consequence of the fact that our laboratory uses centrifugation times that are longer (30 min instead of 20 min), speeds that are faster (500*g* as opposed to 300*g*) and PureSperm® concentrations that are higher (45/90% compared with 40/80%) than those recommended by the manufacturer (to enhance the yield of high-quality spermatozoa), the above experiment was repeated, using swim-up spermatozoa as the negative control. Within 30 min of preparation, the levels of 8OHdG formation in the spermatozoa were assessed and found to be significantly higher in the PureSperm® preparations compared with the swim-up controls (16.1 $\pm$ 3.03%) regardless of whether our laboratory's protocol (36.8 $\pm$ 5.7%; $P < 0.01$) or the manufacturer's (38.2 $\pm$ 7.8%; $P < 0.05$) was used as the sperm preparation method.

In order to confirm that the 8OHdG formation was a consequence of the medium used for selection rather than the act of selection itself, a direct comparison was conducted of unselected spermatozoa prepared by repeated centrifugation and selected cells prepared with PureSperm® or an electrophoretic sperm isolation procedure that involves no extraneous reagent addition or centrifugation (Ainsworth *et al.*, 2005). For these analyses, samples were prepared as described, resuspended in medium BWW and then analysed within 30 min. The results of this analysis revealed that both PureSperm® and the electrophoretic procedure recovered ~20% of the spermatozoa in the original ejaculates,

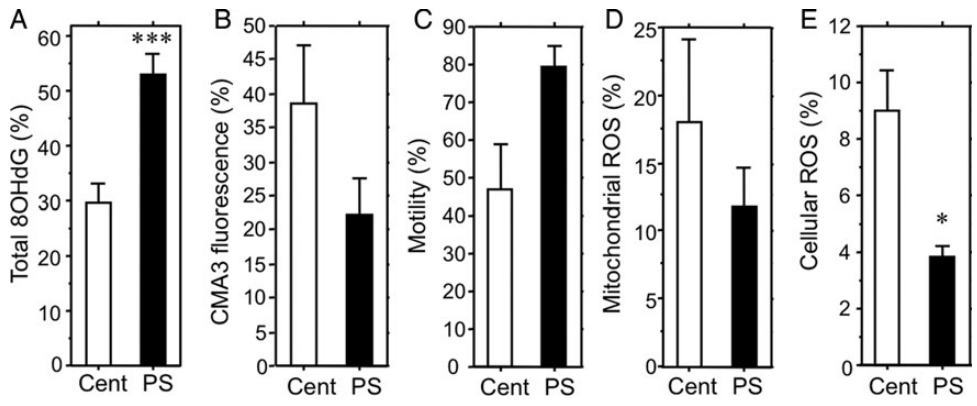

Figure 1 Impact of sperm preparation technique on sperm quality and ROS generation: (**A**) PureSperm® (PS) generated significantly higher levels of 8OHdG formation than spermatozoa prepared by repeated centrifugation (Cent). This difference was not related to any significant changes in chromatin compaction as detected by (**B**) CMA3, (**C**) motility or (**D**) mitochondrial ROS generation; (**E**) total cellular ROS was significantly higher in the cells prepared by repeated centrifugation. ***$P < 0.001$; *$P < 0.05$ according to the paired *t*-test; $n = >4$ for all groups.

whereas the recovery rate for the non-selective repeated centrifugation technique averaged 70–80% (Fig. 2A). The ability of both PureSperm® and electrophoresis to purify sperm suspensions was indicated by highly significant reductions in the number of round cells (Fig. 2B) and significant increases in sperm vitality (Fig. 2C). The electrophoretic system represented a significant improvement over the other techniques in terms of progressive motility (Fig. 2D), STR (Fig. 2E), LIN (Fig. 2F) and percentage rapid (Fig. 2G), although no significant differences were evident for average velocity parameters (VCL, VAP, VSL) or ALH (Supplementary data, Fig. S2). When the same cohort of 41 samples was examined for 8OHdG, the PureSperm® prepared samples were found to have significantly elevated oxidative DNA damage ($P < 0.05$; Fig. 3A) as well as increased TUNEL signals relative to CS10-isolated spermatozoa ($P < 0.01$; Fig. 3B). However, we could find no evidence of increased ROS generation in these PureSperm® prepared samples, indeed the DHE signal was significantly higher in the CS10 preparations ($P < 0.05$; Fig. 3C), while the MSR signal was equivalent across all three preparative methods (Fig. 3D).

Mechanisms by which colloidal silicon preparations influence DNA integrity

In order to determine whether the impact of PureSperm® on oxidative DNA damage was due to exposure to the colloidal silicon solution or required physical centrifugation through this medium, an experiment was conducted in which spermatozoa were prepared by 3 × centrifugation and then either incubated with three concentrations of PureSperm® (80, 90 and 100%) for 45 min at 37°C or centrifuged through this medium for the same period of time. At the end of this incubation period, all samples were centrifuged for 5 min at 500*g* and the spermatozoa recovered. The results depicted in Fig. 4 demonstrated that PureSperm® induced a dose-dependent increase in 8OHdG, irrespective of whether the cells were incubated with this medium or experienced a prolonged centrifugation through it (Fig. 4A). The induction of oxidative DNA damage was not accompanied by any evidence of chromatin instability as measured by SCSA (Fig. 4B), any change in mitochondrial ROS (Fig. 4C) or sperm motility (Fig. 4D).

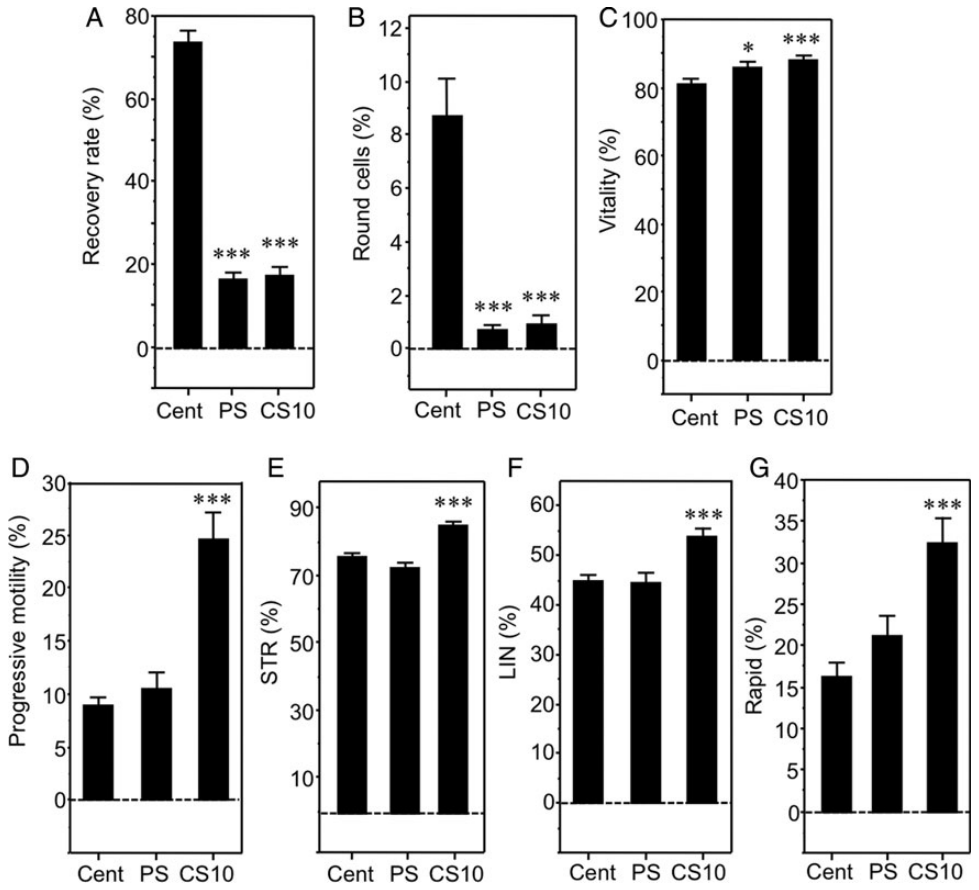

Figure 2 Comparison of three methods for the preparation of human spermatozoa. These methods were based on repeated centrifugation, fractionation of discontinuous PureSperm® gradients and electrophoretic isolation on a CS10 system (CS10). (**A**) An analysis of recovery rates demonstrated that both selective techniques resulted in the recovery of ~20% of the sperm population compared with >70% for repeated centrifugation; (**B**) the selective techniques were also equally efficient in eliminating round cells from the sperm suspensions and (**C**) both selective techniques significantly enriched for sperm vitality. However, the percentage of cells that were (**D**) progressively motile or exhibiting high levels of (**E**) straightness (STR), (**F**) LIN and (**G**) rapid movement were significantly elevated in the electrophoretically prepared cells using the CS10 system. ***$P < 0.001$; *$P < 0.05$ according to analysis of variance (ANOVA) incorporating *post hoc* testing with Fisher's PLSD; $n = 42$.

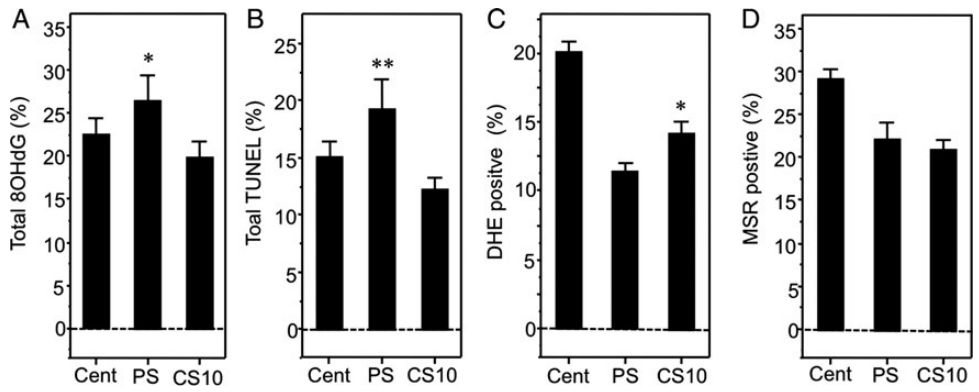

Figure 3 Oxidative stress and DNA damage following preparation of human spermatozoa with different techniques. The latter included repeated centrifugation, fractionation of discontinuous PureSperm® gradients and electrophoretic isolation on a CS10 system. (**A**) 8OHdG expression was significantly elevated following preparation of the spermatozoa on PureSperm® gradients ($n = 42$); (**B**) DNA damage according to the TUNEL assay was also significantly elevated following isolation on PureSperm® gradients ($n = 41$). This DNA damage was not associated with an increase in ROS generation whether monitored by (**C**) DHE ($n = 41$) or (**D**) MSR ($n = 41$). *$P < 0.05$, **$P < 0.01$ according to ANOVA incorporating *post hoc* testing with Fisher's PLSD. (DHE, dihyroethidium; MSR, MitoSOX Red™.)

Importance of metal contamination

From the above results, it was clear that exposure to PureSperm® could induce the formation of 8OHdG adducts. Since the oxidative stress generated under these circumstances did not appear to be related to an increase in ROS generation, an alternative mechanism was proposed by which 8OHdG formation might be induced, involving the localized action of transition metals such as Fe and Cu. The ionic compositions of PureSperm® and BWW were therefore determined by ICP-MS and the results are presented in Table I. From this analysis, it is clear that these fluids varied dramatically in their ionic composition with significantly more Fe, Al, Cr, Cd, Ba and Pb in PureSperm® than medium BWW. Several of the metals highly represented in PureSperm® are known to induce DNA damage via mechanisms involving oxidative stress including Fe, Cu, Al, Pb and Cd (Lankoff *et al.*, 2006; Yedjou *et al.*, 2010; Qiao and Ma, 2013), while others, particularly Zn, tend to exhibit a protective role (Ho, 2004). In order to determine whether the balance of pro- and antioxidant metals in PureSperm® could account for the DNA damage seen in the presence of this sperm preparation medium, medium BWW was modified to contain the following ions at concentrations reflecting the top of the ranges seen in PureSperm®: Al(III) (93.00 mg/l), Fe (II) (40.00 mg/l), Zn (II) (0.28 mg/l), Cu (II) (0.05 mg/l, Cd (II) (0.045 mg/l) and Pb (II) (0.03 mg/l) as the sulphate or, in the case of Pb and Cd, as the nitrate and chloride, respectively. Unfractionated spermatozoa were prepared by repeated centrifugation ($3 \times 500g$ for 5 min) and then incubated with either conventional BWW/PVA medium, as a negative control, or serial dilutions of the ion supplemented BWW medium described above (20–100%). The incubation times selected for this analysis were 1, 2 and 24 h and the results are presented in Fig. 5. Metal ion supplementation induced a dose-dependent decline of sperm motility ($P < 0.001$), which was clearly evident within 1 h (Fig. 5A). Metal supplementation also induced a dose-dependent decline in sperm viability, which was also detectable within 1 h, particularly at the higher levels of ion supplementation (Fig. 5B). The presence of these metals also had a dramatic effect on the levels of 8OHdG formation ($P < 0.001$), such that even at the lowest doses of ion supplementation assessed, representing ~20% of the levels in neat PureSperm®, ~90% of the spermatozoa were 8OHdG positive within the first hour of exposure (Fig. 5C). This oxidative DNA damage was associated with a clear dose-dependent increase in DNA fragmentation as measured with a TUNEL assay ($P < 0.001$) and again this effect was evident within the first hour of exposure (Fig. 5D).

If transition metals are involved in the induction of 8OHdG formation in human spermatozoa, then their redox status will be critical. Transition metals are particularly active in the reduced state since in this form they can support the induction of DNA damage via Fenton chemistry. In light of these considerations we examined the impact of a mild-reducing agent, ascorbic acid, on the induction of DNA damage by PureSperm®. As depicted in Fig. 6, addition of ascorbate to spermatozoa incubated in the presence of PureSperm® resulted in a highly significant increase in oxidative DNA damage ($P < 0.001$) in the absence of any significant change in vitality or motility (Fig. 6A–C).

Conversely, if transition metals are involved in the induction of DNA damage by PureSperm®, then the impact of this sperm preparation medium should be reversed by the addition of chelating agents. In this case, we were able to demonstrate that the addition of EDTA to suspensions of human spermatozoa incubated for 1 h with PureSperm® completely reversed the ability of the latter to both suppress motility (Fig. 6D) and induce DNA damage (Fig. 6E) without having any impact on sperm vitality (Fig. 6F).

In order to determine whether the risk of metal contamination, particularly transition metals, was confined to PureSperm® or was a general feature of colloidal silicon preparations from other manufacturers, a variety of commercially available density centrifugation media were assessed and the results are depicted in Supplementary data, Table S2. It is evident from this table that all of the commercial colloidal silicon preparations examined (AllGrad® 100%, Sil-Select Plus™ Lower Layer, Sil-Select™ Plus Upper layer and SpermGrad™) were significantly

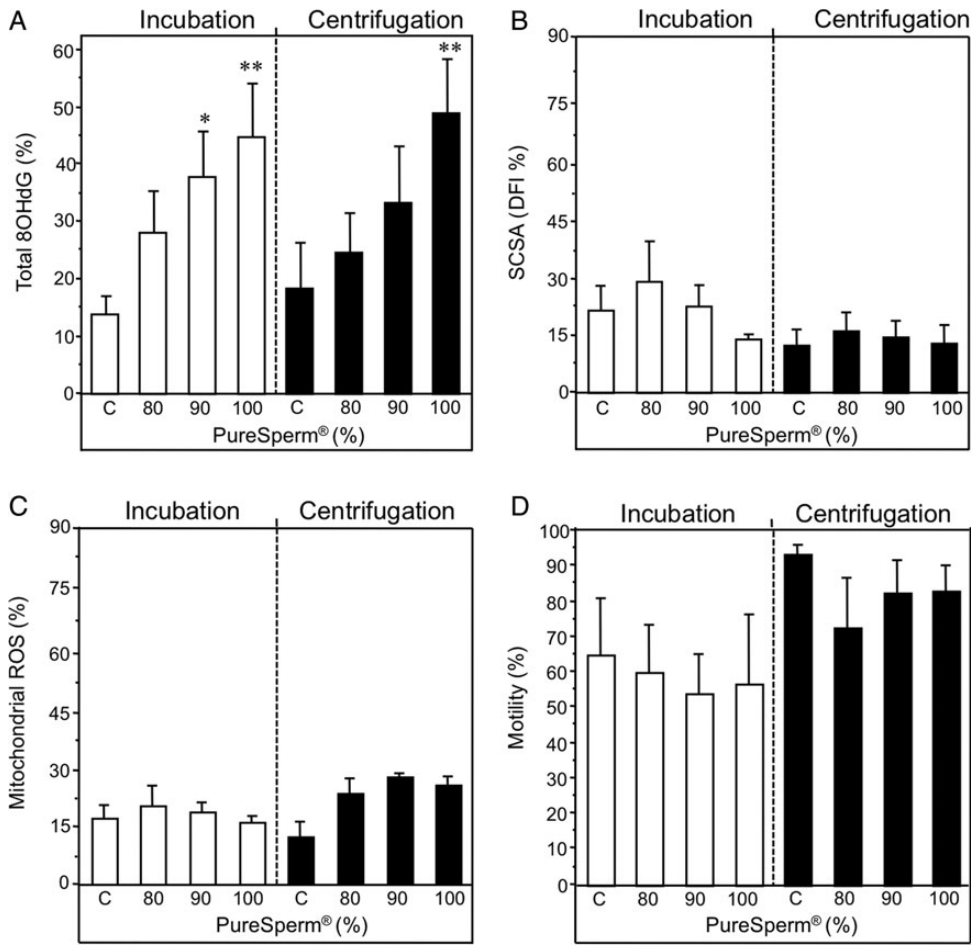

Figure 4 Analysis of the impact of incubation or centrifugation on the induction of oxidative DNA damage. In this experiment spermatozoa were either incubated in the presence of PureSperm® for 45 min at 37°C or centrifuged at 500*g* for 30 min followed by a second centrifugation for 15 min in medium BWW to wash the cells. (**A**) Regardless of whether exposure to PureSperm® did or did not involve the shearing forces associated with centrifugation, there was a dose-dependent increase in oxidative DNA damage; (**B**) this increase in oxidative DNA damage was not associated with any changes in the stability of sperm chromatin as measured by SCSA assay, (**C**) the generation of mitochondrial ROS or (**D**) or the level of sperm motility. *$P < 0.05$; **$P < 0.01$ according to ANOVA incorporating *post hoc* testing with Fisher's PLSD; $n = 6$. (SCSA, sperm chromatin, structure assay.)

($P < 0.001$) contaminated with Fe, at levels that were very similar to those observed in preparations of PureSperm® in the case of AllGrad® and Sil-Select Plus™. SpermGrad™ was also significantly contaminated with Fe but at levels that were significantly ($P < 0.001$) reduced compared with the other colloidal silicon media. Cu was also found in these sperm preparation media but at concentrations that were less than the batch of BWW analysed in this data set (Supplementary data, Table S2). Importantly, these commercial media were also found to be significantly contaminated with Al, Cr, Cd, Ba, Mn, V and Pb (Supplementary data, Table S2) all of which have a history of participation in chemical reactions leading to oxidative stress.

Discussion

Centrifugation of human spermatozoa through discontinuous colloidal silicon gradients, such as Percoll or PureSperm® is known to result in the isolation of spermatozoa that exhibit improved morphology and motility compared with repeated centrifugation protocols (Morrell *et al.*, 2004). Furthermore, such gradients are known to effectively separate high-quality spermatozoa from free radical generating leucocytes and in this manner reduce the levels of oxidative stress associated with sperm preparation (Aitken and Clarkson, 1988). Given the ability of such discontinuous gradients to achieve high levels of cellular purity, it was surprising to observe that these fractionated sperm populations were also characterized by increased levels of oxidative DNA damage and DNA fragmentation as measured by 8OHdG and TUNEL assays (Fig. 3).

The apparent induction of DNA damage when spermatozoa are prepared on discontinuous colloidal silicon gradients has been observed previously on independent occasions (Zini 1999; Stevanato *et al.*, 2008; Aitken *et al.*, 2010). However, such findings are not consistent because others have provided convincing evidence that the isolation of high-quality spermatozoa on discontinuous density gradients generated with colloidal silicon solutions is associated with low levels of DNA

Table I Ionic composition of PureSperm® and BWW.

Metal	Medium BWW	PureSperm®
Fe	0.114 ± 0.029	38.293 ± 7.276**
Mn	0.063 ± 0.054	0.130 ± 0.023
Mg	32.294 ± 4.833*	13.768 ± 1.937
Al	0.012 ± 0.006	68.85 ± 13.69**
V	0.006 ± 0.002	0.004 ± 0.001
Cr	0.007 ± 0.004	0.100 ± 0.009***
Co	0.010 ± 0.003	0.009 ± 0.001
Ni	0.026 ± 0.014	0.103 ± 0.062
Cu	0.009 ± 0.005	0.035 ± 0.018
Zn	0.198 ± 0.076	0.288 ± 0.040
As	0.087 ± 0.052	0.037 ± 0.027
Se	0.124 ± 0.058	0.064 ± 0.022
Sr	0.049 ± 0.032	0.095 ± 0.013
Mo	0.010 ± 0.006	0.006 ± 0.006
Cd	0.000 ± 0.000	0.014 ± 0.003**
Ba	0.004 ± 0.001	0.249 ± 0.035**
Pb	0.004 ± 0.001	0.026 ± 0.004**
Bi	0.002 ± 0.001	0.001 ± 0.001

All data are presented as the mean value ± SEM. Units in mg/l.
***$P < 0.001$, **$P < 0.01$. $n = 3$ independent samples.

damage in the spermatozoa (Donnelly *et al.*, 2000; Sakkas *et al.*, 2000; Morrell *et al.*, 2004). This study therefore set out to determine the source of such inconsistent increases in DNA damage and to determine pathways by which it might be avoided.

Since previous studies have shown that the physical shearing forces associated with sperm centrifugation can trigger ROS generation (Aitken and Clarkson, 1988; Zalata *et al.*, 1995), the importance of such forces in the aetiology of PureSperm®-induced DNA damage was investigated. This analysis demonstrated that prolonged centrifugation was irrelevant, because simply incubating spermatozoa in the presence of PureSperm® was sufficient to induce a dose-dependent increase in oxidative DNA damage irrespective of whether the spermatozoa had been centrifuged during the exposure or not (Fig. 4). Furthermore, the induction of DNA damage was not associated with increased ROS generation or a global increase in lipid peroxidative damage as measured by 4HNE expression and did not involve changes in chromatin stability as measured by the SCSA assay (Figs 1 and 4).

Whatever factors were involved in eliciting DNA damage in the presence of PureSperm® were therefore capable of inducing localized oxidative lesions in the sperm DNA without stimulating a significant global increase in lipid peroxidation. These observations led us to examine the potential importance of metal contamination in generating these effects since cations, such as Fe and Cu, have an affinity for nucleic acids and are capable of inducing oxidative DNA damage and strand breakage as a consequence of Fenton chemistry and the highly localized induction of hydroxyl radical ($OH^{\bullet}$) formation (Lloyd and Phillips, 1999). The latter is known to attack both the sugar and the base elements of the DNA backbone *in situ*, producing strand breaks and oxidative base modifications including 8OHdG formation. An analysis of the ionic composition of PureSperm® was therefore undertaken with surprising results (Table I). This sperm preparation medium contains significantly elevated quantities of Fe, which is known to promote DNA damage in spermatozoa (Moriwaki *et al.*, 2008; Mitchell *et al.*, 2011). Interestingly, PureSperm® was also significantly contaminated with Al and this metal is known to potentiate the oxidative stress associated with exposure to transition metals such as Fe and Cu (Becaria *et al.*, 2003). PureSperm® also contained elevated amounts of Cr, Cd and Pb, which are known to induce DNA damage in various cellular systems (Jomova and Valko, 2011; Qiao and Ma, 2013). Furthermore, Mn and V were also significantly increased relative to BWW in many of the sperm preparation media (Supplementary data, Table S2) and both of these elements are known to be involved in the creation of oxidative stress in different tissues (Valko *et al.*, 2006; Martinez–Finley *et al.*, 2013). In combination, these metals would be expected to induce localized oxidative DNA damage and strand breakage via the creation of $OH^{\bullet}$ radicals and the depletion of protective thiols from protamines in the immediate vicinity. In order to demonstrate this point, medium BWW was modified to contain a selection of the metals (Fe, Cu, Al, Zn, Cd and Pb) typical of neat PureSperm®. Because this density centrifugation medium is normally used at dilutions of 40–45 and 80–90% to create the discontinuous gradients employed in sperm isolation, this metal-supplemented medium was serially diluted to generate a range of concentrations that spermatozoa might be expected to encounter during routine semen preparation (20, 40, 60, 80 and 100%). Spermatozoa incubated in the presence of these metals exhibited a dramatic loss of motility and vitality and an accompanying increase in 8OHdG formation and DNA strand breakage within 1 h, as depicted in Fig. 5. As for the duration of exposure, the period of time when spermatozoa are normally centrifuged through PureSperm® is typically 20–30 min; however, the total period of exposure, from the moment the semen is added to the top of the gradient to the time they are pelleted and washed free of the centrifugation medium, can be as long as 45–60 min, depending on the number of samples being processed at one time.

The dramatic effect of these metals was unexpected because even though oxidative DNA damage and motility loss have been observed following exposure to PureSperm®, such detrimental changes are inconsistently observed and rarely attain the high levels seen in Fig. 5C. The answer to this conundrum is presented in Fig. 6E–F. Thus, the impact of exposure to PureSperm® on motility and 8OHdG formation could be completely negated by the incorporation of the divalent cation chelator, EDTA. According to the manufacturer's product description, PureSperm® is normally supplemented with an unspecified amount of EDTA, which is presumably present to compensate for the presence of transition metals in this medium. The protection afforded by the inclusion of this chelator is however inconsistent for two reasons. First, the toxicity of these transition metals will depend on their relative redox status. Differences in oxidoreductive state between PureSperm® preparations may well explain the variance in DNA damage observed in independent studies. Under reducing conditions, the presence of Fe(II) or Cu(I) will promote the Fenton chemistry that leads to DNA damage and it is for this reason that the addition of ascorbate significantly promoted 8OHdG formation in the presence of PureSperm®. Secondly, there appears to be significant variation between samples in their susceptibility to transition metal toxicity, for reasons that are currently unclear (e.g. see the differing effects on motility depicted in Fig 1C and 6D).

From a practical perspective, the solution to this problem could involve the addition of supplementary EDTA to PureSperm® gradients

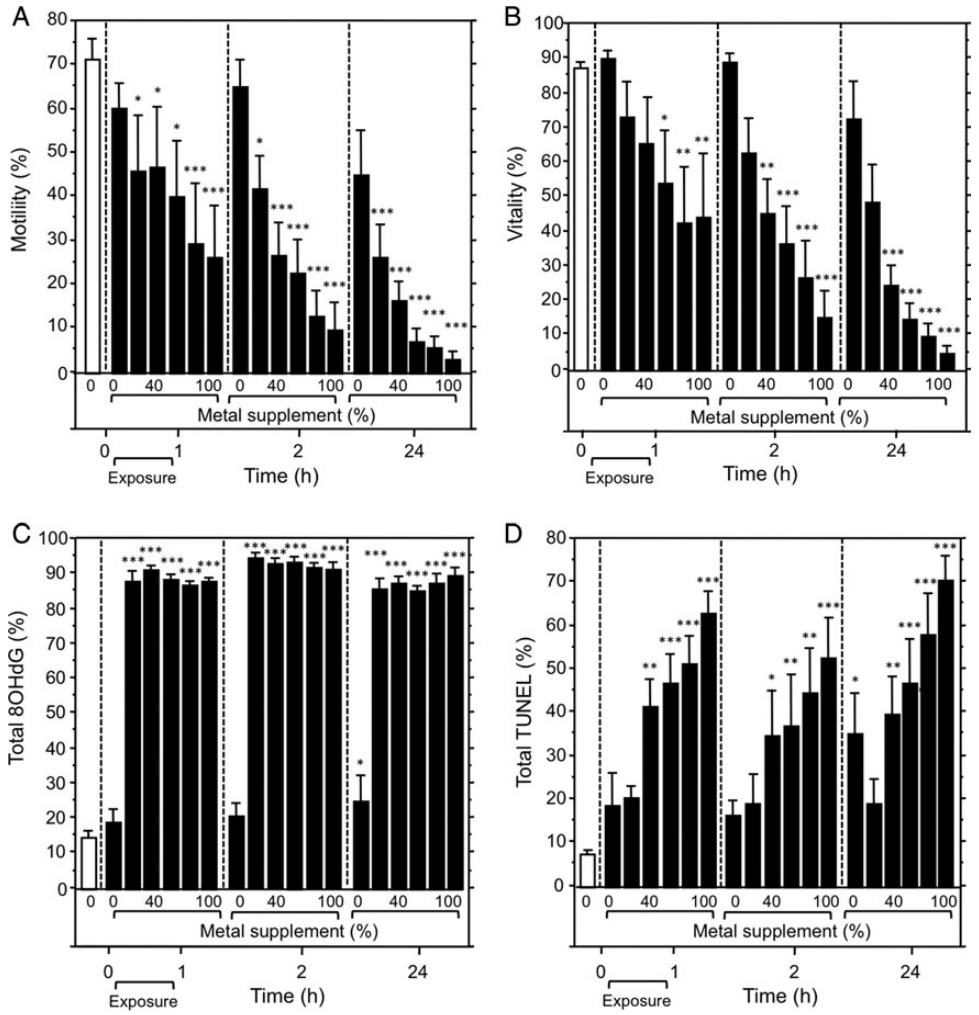

Figure 5 Impact of metal supplementation on the cell biology of human spermatozoa. Unfractionated populations of human spermatozoa were prepared by repeated (×3) cycles of centrifugation (500*g* for 5 min) in medium BWW supplemented with 1 mg/ml PVA. Medium BWW was then supplemented with ions representing the upper range of the concentrations recorded in 100% PureSperm® (Al 93 mg/l, Fe 40 mg/l, Zn 280 μg/l, Cu 52 μg/l, Cd 45 μg/l, Pb 30 μg/l). Spermatozoa at 5×10^6/ml were incubated with serial dilutions of these ions (100, 80, 60, 40, 20%) to reflect the range of exposures that might be encountered during discontinuous density gradient centrifugation. After a 1 h exposure, the spermatozoa were washed in normal medium BWW, resuspended at a concentration of 5×10^6/ml and examined 2 and 24 h post-exposure in order to determine if this combination of metals had triggered changes that would be evident in the short (2 h) or long (24 h) term. (**A**) Metal exposure induced a highly significant, dose-dependent decline in sperm motility ($P < 0.001$) that was already evident within the 1 h exposure period. (**B**) This loss of motility was accompanied by a loss of vitality ($P < 0.001$), (**C**) 8OHdG formation was also significantly ($P < 0.001$) increased by metal exposure, reaching maximal levels at the lowest level of exposure (20%) within the 1 h exposure period; (**D**) this oxidative DNA damage led to a highly significant ($P < 0.001$) increase in TUNEL reactivity that was also evident within the initial 1 h exposure period. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ for differences with the starting values depicted in open bars, according to ANOVA incorporating *post hoc* testing with Fisher's PLSD; $n = 6$. (PVA, polyvinyl alcohol.)

to inhibit $OH^{\bullet}$ formation (Meneghini and Hoffmann, 1980). It may even be possible to improve on EDTA in this context using more powerful iron chelators (Mello Filho *et al.*, 1984). The fact that all of the commercial media tested exhibited significantly elevated levels of metals that are known to precipitate DNA damage (Fe, Mn, Al, V, Cr, Sr Cd and Pb) suggests that this form of sperm preparation should be avoided if possible and alternative sperm preparation methods sought and validated that do not involve exposing these cells to extraneous reagents. In this context both electrophoretic sperm isolation (Ainsworth *et al.*, 2005; Aitken *et al.*, 2011) and swim-up/swim down (Aitken and Clarkson, 1988; Henkel, 2012) techniques may be useful, depending on the intrinsic level of motility. Whatever methods are employed, it is important that we recognize the potential damage that can be inflicted by certain metals on sperm DNA integrity and attempt to eliminate them from the protocols employed in preparing spermatozoa for assisted reproduction technology (ART) purposes.

Finally, it should be emphasized that while this article highlights a potential mechanism by which DNA damage might be induced in human spermatozoa prepared for ART on discontinuous colloidal silicon gradients, there is as yet no direct evidence that Puresperm®, or any related

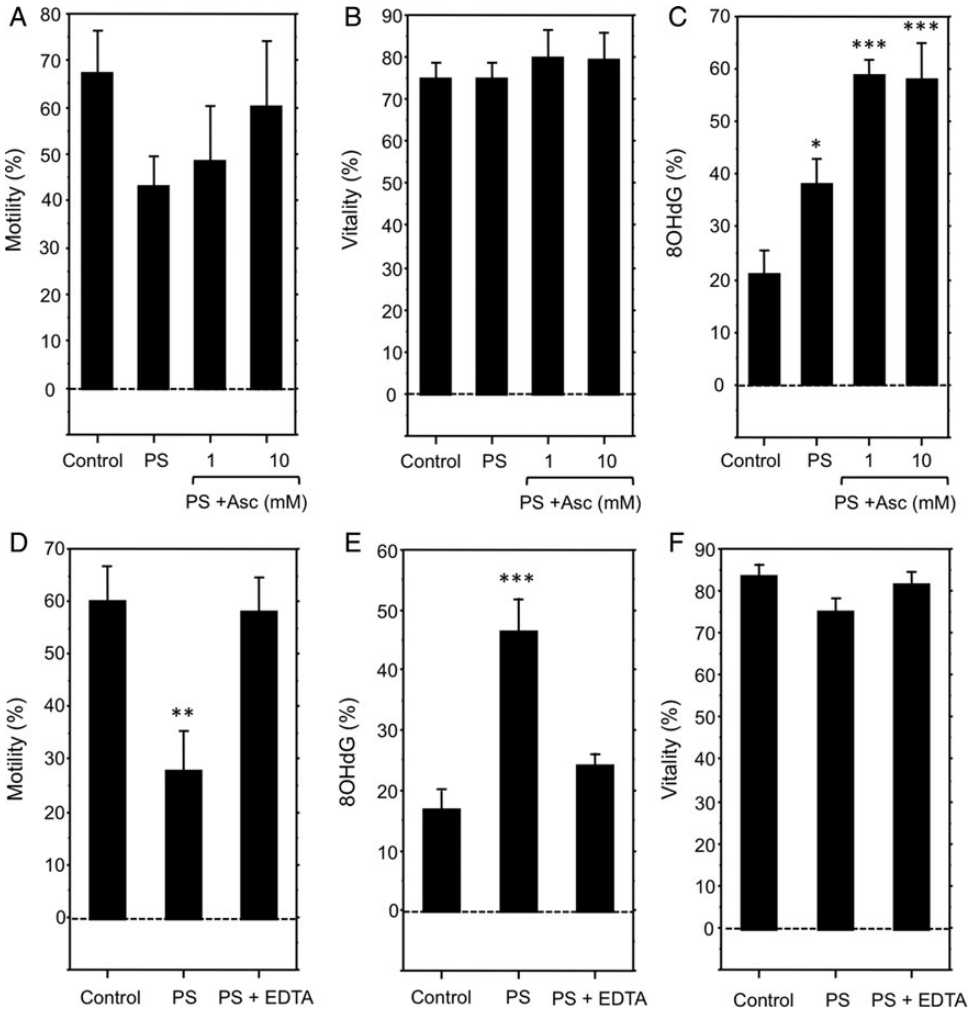

Figure 6 Impact of a reducing agent, sodium ascorbate and EDTA on the levels of DNA damage observed in response to PureSperm®. Addition of 1 or 10 mM ascorbate to unfractionated suspensions of human spermatozoa prepared by repeated centrifugation and resuspension in medium BWW and incubated in the presence of PureSperm® for 1 h did not influence (**A**) sperm motility or (**B**) vitality but (**C**) led to a highly significant increase in the levels of oxidative damage observed in these cells ($P < 0.001$). (**D**) Chelation of metal contaminants with 2 mM EDTA completely reversed the inhibitory effect of PureSperm® on motility and (**E**) 8OHdG formation, while (**F**) vitality remained unchanged. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ according to ANOVA incorporating *post hoc* testing with Fisher's PLSD; $n = 4$.

product, negatively influences the safety of ART. In order to address this question, costly, large-scale studies would have to be conducted in which the outcomes of IVF or ICSI cycles involving spermatozoa prepared either by centrifugation through discontinuous colloidal silicon gradients or alternative methods such as 'swim-up' are compared. No such studies have been conducted and as a result, the clinical significance of the DNA damage induced during sperm preparation is uncertain. Nevertheless, the large volume of literature suggesting that DNA damage in the germ line has negative impacts on such end-points as the normality of embryonic development and the risk of miscarriage (Aitken *et al.*, 2009) should alert us to the need to avoid such damage in the name of 'best practice'.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

R.J.A. conceived the study, analysed the results and drafted the manuscript; J.M.F., L.M., S.W. and H.S.C. prepared the sperm samples and conducted the DNA damage analyses; L.K. conducted the comparison of density gradient centrifugation with electrophoretic sperm separation; T.B.R conducted the mass spectrometry analyses; G.N.D. provided

advice on aspects of DNA damage and all authors read and edited the approved manuscript.

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Conflict of interest

R.J.A. has previously held consultantship with NuSep in the area of sperm preparation technology. He has no current financial interest in this area.

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