Electrophoreotic sperm isolation: optimization of electrophoresis conditions and impact on oxidative stress

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BACKGROUND: The purpose of this study was to optimize the electrophoretic conditions that should be used for the effective isolation of functional human spermatozoa and to determine whether this method of isolating cells was associated with oxidative stress and DNA damage.

METHODS: Human spermatozoa were prepared by repeated centrifugation, discontinuous density gradient centrifugation and electrophoresis followed by assessments of sperm quality.

RESULTS: Systematic analysis of optimal electrophoresis conditions demonstrated that field strength was positively correlated with sperm recovery rates but negatively correlated with sperm movement, irrespective of whether the current or the voltage was held constant. This loss of functionality observed at high power settings was not associated with a major increase in superoxide generation or the induction of oxidative DNA damage. In contrast, discontinuous Percoll gradient centrifugation was shown to produce a significant rise in oxidative DNA base adduct expression in live cells ($P < 0.05$). As a result of these analyses, optimized electrophoretic conditions were defined that permitted sperm recovery rates of around 20%. These electrophoretically isolated cells were not only free of oxidative stress but exhibited significantly enhanced motility ($P < 0.01$) and vitality ($P < 0.001$) compared with the original samples.

CONCLUSIONS: We conclude that while field strength is positively correlated with sperm recovery rates; it is negatively associated with sperm motility. Optimized conditions are described that represent a balance between these opposing forces and permit the isolation of highly motile, vital sperm populations, free from the oxidative DNA damage associated with conventional density gradient centrifugation technologies.

Key words: human spermatozoa / sperm preparation / oxidative stress / DNA damage / electrophoresis

Introduction

Male infertility is a relatively common condition that affects approximately 1 in 20 of the male population (McLachlan and de Kretser, 2001). Although a majority of such patients produce sufficient numbers of spermatozoa to achieve conception, fertility is compromised because these cells are functionally deficient (Hull et al., 1985). This lack of sperm quality can influence both the fertilizing potential of the spermatozoa and their ability to promote normal embryonic development as a consequence of high levels of DNA damage in the paternal genome (Lewis and Aitken, 2005; Aitken et al., 2009). Although some of the physiological, environmental and lifestyle drivers that promote defective sperm function have been elucidated, the root causes of the impaired spermatogenesis observed in such patients are only just beginning to be unravelled. To date, the major factors that have been identified to contribute to poor semen quality and sperm DNA damage include paternal age (Singh et al., 2003; Schmid et al., 2007), diabetes (Akbaje et al., 2008), obesity (Chavarro et al., 2010), radiotherapy (Smit et al., 2010), chemotherapeutic drugs (O’Flaherty et al., 2010) and exposure to lifestyle factors such as cigarette smoke, radiofrequency electromagnetic radiation and alcohol (Aitken et al., 2009; De Iuliis et al., 2009a; Pacey, 2010). The mechanisms by which such factors compromise the functionality of human spermatozoa are thought to include the induction of oxidative stress and DNA damage.
stress as a result of the excessive generation of reactive oxygen species (ROS) and the consequent induction of oxidative damage to the DNA, lipids and proteins that comprise these highly specialized cells (Gomez et al., 1998; De Iuliis et al., 2009b; Aitken and De Iuliis, 2010). Support for this oxidative stress hypothesis has come from numerous studies emphasizing that the spermatozoa of infertile patients show significantly higher levels of ROS generation, lipid peroxidation and oxidative DNA damage than their fertile counterparts (Jones et al., 1979; Aitken and Clarkson, 1987; Alvarez et al., 1987; Engel et al., 1999; Agarwal et al., 2008; Tremellen, 2008; De Iuliis et al., 2009b).

While a majority of the oxidative stress observed in the germ line is caused by a complex mixture of genetic and environmental factors that are, as yet, unresolved and uncontrolled, it is an unfortunate fact that some of this stress is iatrogenically induced during the processing of spermatozoa for assisted reproduction technologies (ARTs). In particular, the centrifugation of unfractionated sperm suspensions in the absence of seminal plasma protection, as might occur when spermatozoa are swum up from a washed pellet, can seriously impair sperm quality through the creation of oxidative stress (Aitken and Clarkson, 1988). To a large extent, this stress arises because of the inevitable presence of leukocytes in unfractionated human sperm suspensions that are typically generating ROS and thus capable of damaging spermatozoa in the immediate vicinity, particularly if no antioxidant protection is provided in the incubation medium (Baker et al., 1996; Twigg et al., 1998). It has also been observed that the physical trauma associated with sperm centrifugation will activate ROS production by these cells (Aitken and Clarkson, 1988; Shekarriz et al., 1995) and induce oxidative DNA damage (Aitken et al., 2010). In order to avoid such damage, it is important that alternative methods are sought for the preparation of human spermatozoa that do not involve the physical shearing forces associated with multiple rounds of centrifugation. One such technique is the electrophoretic sperm isolation procedure described by Ainsworth et al. (2005). In the present study, we report on the optimization of this sperm isolation technique and examine its ability to prepare spermatozoa free from the oxidative stress associated with the conventional methods of sperm preparation for ART.

Materials and Methods

Chemicals

Except where otherwise noted, all chemicals used during this study were of BDH AnalaR brand, purchased from Biolab (Australia) or Sigma (MO, USA).

Semen samples

Semen samples were obtained from volunteer donors with their informed consent. A majority of these subjects were of unknown fertility status and were sourced from the sperm donation programme operated by the Priority Research Centre for Reproductive Science within the University of Newcastle, NSW, Australia. Semen samples were donated by masturbation into a sterile container after a minimum 48 h period of abstinence, and provided to the laboratory staff within an hour of donation. Scientific use of these samples for research purposes was approved by the Human Ethics Committee of the University of Newcastle and the State Minister for Health.

Sperm analyses

Motility assessments were performed on prewarmed (37°C) slides using phase contrast optics on a Zeiss Axioskop 2 microscope at ×400 magnification. The percentage of motile cells (percentage of cells exhibiting any form of flagellar movement) and the percentage of cells exhibiting space-gaining motility (percentage of cells in which the flagellar wave was sufficiently co-ordinated to generate forward movement) were determined as described by O’Connell et al. (2002). Cell vitality assays were conducted using the eosin exclusion test (World Health Organization, 1999). The flow cytometry assays described later also incorporated assessments of cell vitality using Sytox green (SyG; Molecular Probes, CA, USA).

Discontinuous gradient density centrifugation

Purification of human spermatozoa was achieved using a 45 and 90% discontinuous Percoll (GE Healthcare, Castle Hill, Australia) centrifugation gradient, as described (Mitchell et al., 2011). For this procedure, Percoll (90 ml) was supplemented with 10 ml of 10× Ham’s F10 solution, 370 μl sodium lactate syrup, 3 mg sodium pyruvate, 210 mg sodium hydrogen carbonate and 100 mg polyvinyl alcohol (PVA). This isotonic Percoll solution was diluted with HEPES-buffered Biggers, Whitten and Whittingham medium (BWWW) supplemented with 1 mg/ml PVA and maintained at an osmolarity of 300 mOsm/kg (Biggers et al., 1971). Up to 3 ml of neat semen were layered on the top of each gradient and centrifuged at 500g for 30 min. Following centrifugation, the seminal plasma and Percoll were removed and discarded. Purified spermatozoa were recovered from the base of the 90% Percoll fraction and resuspended at a concentration of 6 × 10^6 cell/ml in BWWW.

Electrophoretic sperm isolation

The prototype CS-10 electrophoretic sperm isolation device is depicted in Fig. 1A (Ainsworth et al., 2005). The separation unit consists of two chambers, each exhibiting a capacity of 400 μl, separated by a polycarbonate filter with a pore size of 5 μm. The separation cassette is bounded by polyacrylamide restriction membranes (10 kDa) that permit the flow of electrolytes but restrict the passage of cells and large proteins. Buffer (10 mM HEPES, 30 mM NaCl and 0.2 M sucrose, pH 7.4) is circulated in the space between the electrodes and the restriction membranes (Fig. 1B). All separations were performed at 25°C. For step-wise voltage trials, semen samples were subjected to five, 5-min runs at varying fixed voltages. Whole semen, 400 μl, was loaded in the inoculation chamber of the CS-10 with 400 μl standard CS-10 buffer in the separation chamber and 60 ml standard CS-10 buffer in the running buffer reservoir. Electrophoresis was undertaken for 5 min at 9, 12, 15 and 18 V. Electric current was applied using a Bio-Rad Power Pac 3000 (Bio-Rad, Hercules, CA, USA). Current was monitored and recorded at 1 min intervals throughout all runs. A run with no voltage applied (0 V) was included as a control. At the end of each run, the separated and residual fractions were removed and diluted in BWWW before determining sperm motility, vitality and cell count.

For step-wise current trials semen samples were subjected to six, 5 min runs at varying levels of fixed current. Whole semen, 400 μl, was loaded in the inoculation chamber of the CS-10 with 400 μl standard CS-10 buffer in the separation chamber and 60 ml standard CS-10 buffer in the running buffer reservoir. Electrophoresis was undertaken for 5 min at 50, 65, 80, 95 and 110 mA. Electric current was applied using a Bio-Rad Power Pac 3000. Voltage was monitored and recorded at 1 min intervals throughout all runs. A run with no current applied (0 mA) was included as a control.
At the end of each run, the separated and residual fractions were removed and diluted in BWW before determining motility, vitality and cell count.

**Reactive oxygen species**

Spermatozoa were assessed for ROS generation using the combination of dihydroethidium (DHE) and SyG, as described by De Iuliis et al. (2006). DHE is a weak fluorescent probe that, when oxidized, produces ethidium and 2-hydroxyethidium, both of which intercalate with DNA and strongly fluoresce red/orange (De Iuliis et al., 2006). SyG also stains DNA, and fluoresces green, but cannot cross the intact cell membranes and hence will only stain dead cells. In this staining procedure, cells are incubated with both DHE and SyG simultaneously. Dead cells take up SyG and residual ethidium present in the DHE preparation, and so fluoresce both green and red. Live cells do not take up the SyG and will only stain red if they are actively engaged in ROS generation. SyG and DHE were both obtained from Molecular Probes, supplied as 5 mM solutions in dimethylsulphoxide (DMSO). SyG was further diluted 1 in 40 with DMSO and this stock solution stored at 2-8°C along with the undiluted DHE stock. A DHE/SyG solution was made by adding 1 μl of each of these stock solutions to 248 μl of BWW. A 20 μl aliquot of this DHE/SyG solution was then added to 180 μl of spermatozoa at 5 × 10^6/ml and incubated in the dark at 37°C for 15 min. The cells were then washed by centrifugation (500g for 5 min) and the pellet resuspended in 1 ml BWW. This cell preparation was then assessed immediately by flow cytometry using a Becton-Dickinson fluorescence-activated cell sorter (FACS)-Calibur flow cytometer with a 488 nm argon laser, and the data captured and analysed using CellQuest software (Becton Dickinson CA, USA). A total of 10,000 events were assessed for each sample. Forward scatter (FSC), side scatter (SSC), 530/30 band pass green fluorescence (FL1) and 585/42 band pass red fluorescence (FL2) were all recorded. FSC and SSC parameters were used to generate a scatter plot, which was then electronically gated to exclude larger contaminating cells and ensure that only spermatozoa were assessed for fluorescence. FL1 and FL2 parameters were used to generate a second scatter plot, using only gated data, showing the fluorescence profile of the sample. This was subjected to quadrant analysis such that the upper left quadrant indicated the presence of DHE-positive/SyG negative cells (ROS-generating, live spermatozoa).

Intracellular generation of mitochondrial superoxide anion was determined using MitoSOX Red (MSR: Molecular Probes). For this assay, stock solutions of MSR (5 mM in DMSO) and SyG (0.125 mM in DMSO) were diluted in BWW and added to spermatozoa at 10 × 10^6 cells/ml to give final concentrations of 2 and 0.05 μM, respectively. The spermatozoa were then incubated for 15 min at 37°C, centrifuged for 5 min at 500g, resuspended in 1 ml BWW and then transferred to 5 ml FACS tubes. The MSR (red) and SyG (green) fluorescence was then
measured on a FACS-Calibur flow cytometer with a 488 nm argon laser, as described already. The results were expressed as the percentage of MSR-positive/SyG negative cells (mitochondrial ROS-generating, live spermatozoa).

8-hydroxy-2′-deoxyguanosine
The formation of 8-hydroxy-2′-deoxyguanosine (8-OHdG) was measured using a specific antibody (Biotin OxyDNA test Kit, Biotrin International Ltd., Dublin, Ireland) conjugated to fluorescein isothiocyanate as previously described (Aitken et al., 2010). For the positive control, spermatozoa were incubated for 1 h with H2O2 (2 mM) and CuSO4 (1 mM) in a final volume of 200 μL BWW. The concentration of H2O2 was measured by determining the absorbance of the sample 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). They were subsequently washed 1 × with BWW before being incubated with 2 mM dithiothreitol for 45 min and fixed in 2% paraformaldehyde for 15 min at 4°C. After fixation, the spermatozoa were centrifuged (500g 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 cells were subsequently analysed for 8-OHdG expression as previously described (De Iuliis et al., 2009a,b) using argon laser excitation at 488 nm coupled with emission measurements using 530/30 band pass (green) in 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.

Statistics
All analyses were replicated at least three times and the results were analysed by analysis of variance (ANOVA) followed by a comparison of group means using Dunnnett’s t-test. Nonlinear regression ANOVA was also performed (Zar, 1974). Percentage data were arcsine-transformed prior to regression analysis. Paired comparisons were conducted using the paired t-test and verified using a non-parametric statistic, the Wilcoxon sign rank test. Probabilities of $P < 0.05$ were considered significant.

Results
Optimization of electrophoretic sperm separation
A series of experiments was conducted using ranges of fixed currents or voltages, and then space-gaining motility, total motility, vitality and cell concentration were assessed in order to monitor the efficacy of the separation process. During each run, the varying power parameter (voltage or current) was monitored at 60 s intervals. In order to compare results from the fixed voltage series (in the range 0–18 V) directly with the fixed current series (0–110 mA), the set voltages in the fixed voltage series were replaced by average currents, calculated from the current observations throughout all the runs conducted at that set voltage. Standard errors were also calculated and were included as x-axis error bars in Fig. 2. The observed motility, vitality and cell concentration results for the separated fractions were converted to percentages of the original ejaculate values before calculating the group means. Figure 2A shows that with higher power settings, there was a progressive loss of total motility that was significantly more marked ($P < 0.05$) when the current was fixed, rather than the voltage. When space-gaining progressive motility was evaluated, a similar progressive loss of movement was observed at higher power settings although the statistical comparison of the two regression lines yielded no differences between the fixed voltage and fixed current series (Fig. 2B). Similarly, analysis of sperm vitality revealed no difference between the regression lines fitted to the fixed voltage and fixed current experiments, permitting a single regression line to be drawn through the entire data set (Fig. 2C). The actual number of spermatozoa recovered from the ejaculates increased positively and significantly ($P < 0.001$) with electric field strength (Fig. 2D); however, regression analysis did not reveal any differences between the constant current and constant voltage data sets.

From these data, it is evident that the electrophoretic recovery of human spermatozoa directly from semen increases in direct proportion to the electric field strength, whereas the motility (but not the vitality) decreases. The optimal power setting for sperm separation is therefore a compromise between maximizing the efficiency of sperm recovery and minimizing the degree of motility loss.

Free radicals and motility loss
Since motility loss is known to be mediated by excessive ROS production (Jones et al., 1979; Aitken et al., 1989), the above results raised the possibility that subjecting human spermatozoa to an electric current might have induced a state of oxidative stress. In order to investigate this possibility, human spermatozoa were subjected to prolonged electrophoresis at a constant current of 75 mA in order to establish conditions that would reliably and robustly lead to highly significant ($P < 0.001$) losses of motility and vitality (Fig. 3A). After 10 min electrophoresis, space-gain motility was impaired ($P < 0.01$) while after 20 min electrophoresis, total motility, space-gain motility and vitality were all compromised ($P < 0.01$). If electrophoresis continued for 30 min, then no motility and very little vitality remained in the sperm population (Fig. 3A).

In order to determine whether this loss of functionality on prolonged incubation was mediated by free radicals, ejaculates were electrophoresed for a period up to 25 min and the level of ROS generation monitored with DHE (De Iuliis et al., 2006). Such prolonged electrophoresis predictably impaired sperm motility but had no significant effect on ROS generation (Fig. 3B). Furthermore, the electrophoresis-induced loss of sperm motility (Fig. 3C and D), space-gain motility (data not shown) and vitality (data not shown) were not rescued by the presence of high concentrations (10 mM) of antioxidants in the form of hypotaurine and N-acetylcysteine, that have been shown to protect spermatozoa against oxidative stress (Baker et al., 1996).

Oxidative DNA damage
In order to be certain that the electrophoretic technique did not induce oxidative DNA damage, populations of human spermatozoa were divided into two and prepared by discontinuous Percoll gradient centrifugation or electrophoresis under constant voltage (12 V for 5 min). Under these conditions, both Percoll and the electrophoretic technique enhanced ($P < 0.001$) the proportion of the sperm population classified as alive using SyG as the probe, relative to an unfractionated population prepared by repeated centrifugation (Fig. 4A). However, measurement of the levels of oxidative DNA damage sustained by these cell populations revealed a marked
difference between the Percoll and electrophoretically prepared spermatozoa, with the former exhibiting a doubling of the size of the 8-OHdG-positive population relative to the latter (Fig. 4B; \( P < 0.05 \)). In the same samples, no increase in DHE signal was observed, suggesting that whatever factors are responsible for the increased 8-OHdG formation seen in Percoll-prepared material, a major enhancement of cellular superoxide anion generation does not appear to be involved (Fig. 4C). The MSR results did show a tendency to increase in the Percoll-prepared samples (Fig. 4D); so at this stage we cannot rule out a contribution from the sperm mitochondria to the oxidative DNA damage seen following Percoll preparation; however, in this particular study, the MSR increase did not reach statistical significance. In the context of this paper, the low DHE and MSR signals seen in the electrophoretically prepared material was consistent with the general conclusion that this cell preparation technique is not associated with the cellular generation of oxidative stress.

**Performance of the electrophoretic system**

Although the electrophoretic system seemed to be incapable of inducing a state of oxidative stress, the power settings still had to be optimized in order to maximize sperm recovery rates, while minimizing the risk of motility loss. Such an optimized field strength was found to be around 75 mA (fixed current) or 12 V (fixed voltage) for 5 min. Having established these conditions for electrophoretic separation, a set of samples was subjected to this procedure in order to determine the relationship between semen quality and the attributes of the isolated gametes. Figure 5A reveals that the concentration of spermatozoa isolated in this manner was highly correlated with the concentration of spermatozoa in the original semen sample (\( R^2 = 0.86; P < 0.001 \)). When expressed in terms of sperm recovery rates (i.e. as a percentage of the sperm concentration in the original ejaculate), the range of recoveries was 8.6–28.7% with a mean ± SEM of 17.9 ± 1.3% (Fig. 5B). Similarly, the space-gaining motility of the isolated cells was significantly correlated with the same measure.
of motility in the ejaculate (Fig. 5C; $R^2 = 0.34; P < 0.01$). Expression of the results as a percentage of the space-gaining motility observed in the ejaculate (designated 100%) revealed an increase in this parameter following separation ($P < 0.01$), giving a mean $\pm$ SEM value of $132.6 \pm 10.3\%$. Even though not all samples showed an improvement in their motility following electrophoresis (Fig. 5D), the poorest sample exhibited $82.66\%$ of the motility seen in the original ejaculate, while the best exceeded $250\%$. Similarly, total motility in the electrophoretically isolated sample was correlated with motility in the ejaculate ($P < 0.05$) and was enhanced ($P < 0.01$) following electrophoretic isolation, giving a mean $\pm$ SEM value of $124.4 \pm 6.9\%$. Vitality was uniformly high in both the original ejaculates and the separated samples (Fig. 5E) but was nevertheless significantly enhanced in the separated samples ($P < 0.001$), giving a mean $\pm$ SEM improvement of $117.5 \pm 2.3\%$ compared with the original sample (Fig. 5F).

**Discussion**

One of the major challenges for ART is to devise methods for the preparation of human spermatozoa that are fast, efficient and capable of isolating functional cells in the absence of iatrogenic damage. While density gradient centrifugation and swim-up techniques have been widely adopted by the ART industry, these methods are not beyond criticism. The swim-up technique is dependent on the intrinsic motility of the sperm population and loses efficacy in asthenozoospermic samples characterized by a seriously compromised capacity for movement. Swim-up techniques may also involve the exposure of spermatozoa to prolonged oxidative stress should the semen be significantly contaminated with free radical-generating leukocytes (Aitken and Clarkson, 1988; Baker et al., 1996). Centrifugation through discontinuous density gradients composed of colloidal silica particles coated with silane or polyvinylpyrrolidone overcomes some of these problems and has been widely accepted as the standard method of sperm preparation for assisted conception programmes (Aitken et al., 1998). However, the discontinuous density gradient methods may not be particularly effective in samples possessing small numbers of motile spermatozoa or those that are poorly liquefied (Mortimer, 1994). Furthermore, we have recently shown (and confirmed in the present study) that such techniques can artificially enhance the levels of oxidative DNA damage recorded in the spermatozoa (Aitken et al., 2010).
Electrophoretic sperm separation represents a potential alternative to density gradient centrifugation for the preparation of this material. The method is based on the observation that good quality human spermatozoa are characterized by both a high negative charge, favoring their movement towards the anode, and small size, facilitating their transition across a 5 μm polycarbonate separation membrane (Ainsworth et al., 2005). The method is fast, efficient and effective for complex cellular mixtures, including testicular biopsy material. Furthermore, the spermatozoa isolated with this technique appear to be relatively free from DNA damage (Ainsworth et al., 2005). The clinical utility of this approach was initially demonstrated in a case study of a couple experiencing infertility associated with high levels of DNA damage in the spermatozoa (Ainsworth et al., 2006). More recently, these studies were extended to a limited clinical trial in which the electrophoretic system was shown to be as effective as discontinuous gradient centrifugation, in terms of sperm recovery, fertilization and embryo cleavage and pregnancy rates, while taking a fraction of the time (Fleming et al., 2008).

In this study, we have conducted a detailed analysis of the electrophoretic conditions required for optimal sperm recovery. In essence, this research has demonstrated that sperm recovery rates increased in relation to the electric field strength while sperm motility declined, irrespective of whether the electrophoretic conditions were defined by constant voltage or constant current (Fig. 2). Although total motility (proportion of cells capable of flagellar movement) was slightly favoured by electrophoresis conditions featuring constant voltage, this difference was not apparent when progressive (space-gaining) motility was considered. Since progressive motility is an essential prerequisite for fertility, we conclude that it makes little difference if the voltage or the current is fixed for the electrophoretic run. However, if the electric field is either too strong (Fig. 2B) or maintained for an excessive period of time (Fig. 3A), then sperm motility, and ultimately viability, will be compromised.

Importantly, the loss of functionality associated with exposure to electric current was not associated with an increase in ROS generation (Fig. 3). Furthermore, this method of sperm preparation was not associated with an increase in oxidative DNA base adduct formation (Fig. 4): the latter sets this method apart from discontinuous gradient centrifugation methods, which have been shown to generate significant increases in 8-OHdG formation in the sperm nucleus (Aitken et al., 2010). Furthermore, while density gradient centrifugation is well known for isolating sperm populations with enhanced motility and vitality (Griveau and Lannou, 1994; Mortimer, 1994) paradoxically this technique has also been shown to induce an increase in DNA fragmentation (Zini et al., 1999), in keeping with the enhanced formation of 8-OHdG observed in the present and previous (Aitken et al., 2010) studies. These observations are also consistent with the conclusion drawn by De Iuliis et al. (2009b) that most DNA damage in the male germ line is oxidatively induced.

The cause of this oxidative DNA damage is not known since it does not appear to involve a marked increase in the cellular (DHE) or mitochondrial (MSR) generation of ROS (Fig. 4C and D). One factor to consider is that the generation of ROS during discontinuous density gradient centrifugation might vary markedly between samples (Zini et al., 2000) and would be very dependent on the attributes of the particular samples subjected to analysis. However this would not explain why in the present study, Percoll centrifugation induced oxidative DNA damage in live cells, while ROS generation remained relatively unaffected (Fig. 4B). It is possible that the quantities of ROS needed to trigger 8-OHdG formation and DNA fragmentation are very small or that the species of oxygen metabolite involved in 8-OHdG formation are not detected by the DHE and MSR probes. A suitable candidate molecule in this context would be H2O2, which is clearly capable of inducing 8-OHdG formation and DNA fragmentation (Takeuchi and Morimoto, 1993; Aitken et al., 1998) but would not be detected by the superoxide-anion-dependent probes used in this study. Another possibility is that Percoll somehow

**Figure 4** Impact of sperm preparation protocols on sperm quality. (A) Sperm preparations were either unfractionated (prepared by three cycles of centrifugation (500g) and resuspension in medium BWW (neat)) or were subjected to electrophoretic sperm separation at 12 V for 5 min (ES) or discontinuous Percoll gradient centrifugation (Percoll). A, both ES and Percoll generated sperm populations were significantly enriched with vital cells (**p < 0.001 for comparisons with the neat sample by Dunnett’s t-test). (B) The Percoll preparations were however associated with a significant increase in the percentage of live cells exhibiting high levels of 8-OHdG (*p < 0.05 for the comparison with neat sample by Dunnett’s t-test). (C) The increase in oxidative DNA damage observed following Percoll preparation was not associated with a significant change in either cellular superoxide generation as measured by DHE or; (D) Mitochondrial superoxide generation as measured by MSR.
interferes with the ability of an oxoguanine glycosylase to remove 8-OHdG residues from the sperm nucleus. Further studies will clearly be needed to establish the mechanisms of Percoll-induced 8-OHdG accumulation in sperm.

Whatever be the nature of the oxidative stress associated with density gradient centrifugation, it was not observed during electrophoretic sperm isolation, even under conditions where the field strength or duration of current flow were so excessive that sperm motility and vitality were lost. The loss of sperm movement observed on prolonged electrophoresis is unlikely to have involved any changes in mitochondrial function because glycolysis is the major source of ATP generation in these cells. In fact, oxidative phosphorylation can be completely silenced in human spermatozoa by addition of respiratory chain inhibitors, such as antimycin, rotenone and even potassium cyanide, and no impact on sperm motility is observed in the short-term (Hong et al., 1983). Furthermore, the fact that IVF success rates are no different whether fertilization is achieved with electrophoretically prepared spermatozoa or spermatozoa isolated on discontinuous density gradients indicates that the temporary exposure of these cells to electric current does not compromise their quality.

Figure 5 Performance of the electrophoretic sperm isolation system. (A) The concentration of spermatozoa recovered following electrophoretic separation (ES) was highly correlated with the sperm count in the original ejaculate ($R^2 = 0.86; P < 0.001; n = 17$). (B) Expression of these results in terms of the percentage of cells recovered from the ejaculate gave a mean recovery rate of around 20%. (C) space-gaining, progressive motility in electrophoretically isolated samples was also highly correlated with space-gaining motility in the ejaculate ($R^2 = 0.34; P < 0.01; n = 19$). (D) Expression of these results as a function of the space-gaining motility in the ejaculate revealed a significant enhancement in the isolated cell populations ($P < 0.01$). (E) The vitality of the electrophoretically isolated cells was uniformly high ($n = 19$). (F) Expression of these results as a function of the sperm vitality observed in the original ejaculate revealed a highly significant enhancement in this parameter ($P < 0.001$) following electrophoretic separation.
in any significant way (Fleming et al., 2008). Indeed, the oxidative stress and DNA damage observed following the exposure of human spermatozoa to an electric field are so minimal that this may constitute an effective method for immobilizing spermatozoa for ICSI in addition to its fundamental role as a fast, effective method for the isolation of motile spermatozoa for assisted conception purposes. Further studies will now be needed to evaluate the limits of this system’s performance when presented with the kind of seriously compromised sperm samples found in infertility clinics.

Authors’ roles

R.J.A. conceived the study, helped with the analysis of the data and generated the first draft of the paper. A.R.H. conducted all of the work on the optimization of electrophoretic conditions and studied the impact of antioxidants. L.K. conducted the flow cytometry analyses of reactive oxygen species generation and determined the incidence of oxidative DNA damage. All authors contributed to the final version of the manuscript.

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Conflict of interest

R.J.A. is Chairman of the Scientific Advisory Board for NuSep, a commercial entity with an interest in developing the electrophoretic sperm isolation system to meet the needs of the assisted conception industry.

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