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The effects of antioxidant supplementation during Percoll preparation on human sperm DNA integrity

Ciara M.Hughes[1,3], Sheena E.M.Lewis[1], Valerie J.McKelvey-Martin[2] and W.Thompson[1]

[1]Department of Obstetrics and Gynaecology, The Queens University of Belfast, Institute of Clinical Science, Grosvenor Road, Belfast BT12 6BJ and [2]Cancer and Ageing Research Group, School of Biomedical Sciences, The University of Ulster, Coleraine, UK

[3]To whom correspondence should be addressed

The integrity of sperm DNA is crucial for the maintenance of genetic health. A major source of damage is reactive oxygen species (ROS) generation; therefore, antioxidants may afford protection to sperm DNA. The objectives of the study were, first, to measure the effects of antioxidant supplementation *in vitro* on endogenous DNA damage in spermatozoa using the single cell gel electrophoresis (comet) assay and, second, to assess the effect of antioxidant supplementation given prior to X-ray irradiation on induced DNA damage. Spermatozoa from 150 patients were prepared by Percoll centrifugation in the presence of ascorbic acid (300, 600 μM), alpha tocopherol (30, 60 μM), urate (200, 400 μM), or acetyl cysteine (5, 10 μM). DNA damage was induced by 30 Gy X-irradiation. DNA strand breakage was measured using the comet assay. Sperm DNA was protected from DNA damage by ascorbic acid (600 μM), alpha tocopherol (30 and 60 μM) and urate (400 μM). These antioxidants provided protection from subsequent DNA damage by X-ray irradiation. In contrast, acetyl cysteine or ascorbate and alpha tocopherol together induced further DNA damage. Supplementation *in vitro* with the antioxidants ascorbate, urate and alpha tocopherol separately has beneficial effects for sperm DNA integrity.

Key words: alpha tocopherol/ascorbic acid/comet assay/DNA damage/urate

Introduction

Male infertility accounts for 40% of infertility problems (Fleming *et al.*, 1995). Using classical light microscopy, only a limited diagnosis of subfertility may be made by assessing low sperm concentration or poor motility. As a result, tests have been introduced to determine other aspects of sperm function: quantitative sperm motion (Zhu *et al.*, 1994), sperm–zona interaction (Liu *et al.*,1988), the acrosome reaction (Liu and Baker, 1988) and fusion with the oocyte by the zona-free hamster oocyte penetration assay (Kruger *et al.*, 1988). However with the advent of intracytoplasmic sperm injection (ICSI) (Palmero *et al.*, 1992; Van Steirteghem *et al.*, 1994), many of these sperm parameters have become irrelevant, as it has been possible to fertilize oocytes using spermatozoa with intact acrosomes, no motility, sperm heads without tails and immature spermatozoa (Baker, 1993), and so new tests are needed to assess sperm health. Ideally, such tests should predict the health of the offspring and not just the success of fertilization and/or implantation. Whatever the assisted conception technique, the quality of the sperm DNA is still of paramount importance for accurate transmission of genetic material to the next generation. Damaged DNA may not prevent fertilization from occurring but may lead to fetal abnormalities which will only be apparent later. Infertility may be linked to DNA damage, as the sperm DNA of infertile patients has been shown to be more susceptible to damage *in vitro* than DNA from fertile men (Hughes *et al.*, 1996). The DNA status of individual cells may be determined using the single cell gel electrophoresis (comet) assay (McKelvey-Martin *et al.*, 1993) which has been modified to measure DNA damage in human spermatozoa (Hughes *et al.*, 1996, 1997).

A major source of damage to DNA is by reactive oxygen species (ROS) (Steenken, 1989). It has been shown that spermatozoa are capable of generating ROS such as the superoxide anion (O_2^-) which subsequently forms H_2O_2 under the influence of intracellular superoxide dismutase (Aitken and Clarkson, 1987; Alvarez *et al.*, 1987). In human semen, defective spermatozoa and contaminating neutrophils are also potential sources of the oxidant hydrogen peroxide (Aitken and West, 1990; Aitken *et al.*, 1992; Kessopoulou *et al.*, 1992). Spermatozoa are uniquely susceptible to oxidative damage because of their differentiation (Jones *et al.*, 1973). As spermatozoa discard the majority of their cytoplasm during the final stages of spermatogenesis, they lose most of the cytoplasmic defence enzymes which protect somatic cells from peroxidative damage. The capacity for DNA repair is also lost as mature spermatozoa do not have any repair enzymes (Chandley and Kofman-Alfaro, 1971; Van Loon *et al.*, 1991) making them more vulnerable to damage than any other cell type. The antioxidants that are present in the seminal plasma are therefore an important source of protection.

During sperm preparation techniques for assisted conception, it is necessary to remove the spermatozoa from the seminal plasma and therefore from their antioxidant protection. This leaves the spermatozoa vulnerable to oxidative attack. The present study determines the effects of two sperm preparation techniques on DNA integrity and the role of antioxidant supplements *in vitro* in protecting sperm DNA during preparation and from induced damage. In a previous study (Lewis *et al.*, 1997), we found that ascorbic acid and urate made up most of the chain-breaking antioxidant capacity of seminal

plasma in fertile men; therefore, these antioxidants were used in the present study at similar concentrations. Alpha tocopherol and acetyl cysteine have been found in previous studies (Aitken and Clarkson, 1989; Kessopoulou *et al.*, 1995; Baker *et al.*, 1996) to be of benefit to sperm parameters; therefore these antioxidants were also studied here to assess any beneficial effect on sperm DNA. To ascertain the extent of any protection additional damage was induced by irradiation after supplementation.

Materials and methods

Semen analysis

Semen samples were obtained from 150 patients after recommended abstinence from sexual activity for 3 days, so that spermatozoa from 30 subjects could be studied following treatment with each of the five selected antioxidants. Routine semen analysis was carried out by light microscopy and the percentage normal morphology (Kruger *et al.*, 1987) for each sample was determined. Ten additional samples were used to examine the DNA quality of the spermatozoa following two different preparation techniques; Percoll centrifugation and direct swim-up. Twenty samples were also used to determine at which stage of the protocol the antioxidants exerted their effect.

Sperm preparation

In order to determine whether sperm preparation technique has any effect on sperm DNA quality, 10 samples were each divided into three aliquots and a fraction of each sample was prepared by Percoll, direct swim-up or direct swim-up in the presence of endotoxins (10 ng/ml). This chosen concentration of endotoxins added to the swim-up media is the same as found in Percoll (Sigma Chemical Co., Poole, Dorset, UK). The swim-up process involved layering semen (1 ml) beneath 1 ml of Biggers–Whitten–Whittingham (BWW) medium and incubating the test tube at a 45% angle at 37°C for 30 min, to allow the spermatozoa to swim out of the seminal plasma into the BWW media.

Percoll preparation of freshly liquefied semen by two-step discontinuous Percoll gradient centrifugation was carried out as follows: (i) two-layer Percoll (95.0–47.5%) centrifugation at 500 *g* for 20 min, (ii) concentration step by centrifugation at 250 *g* for 10 min, and (iii) the sperm cells were diluted with BWW medium (Biggers *et al.* 1971) to a concentration of 1×10^5/50 μl.

Antioxidant incubation

Semen from each of the 150 subjects was divided into three aliquots. One control aliquot was then prepared by Percoll as described, while the other two aliquots were prepared by Percoll in the presence of an antioxidant at one of two different concentrations that was added to the Percoll layers and the BWW. Thirty subjects were therefore studied in this manner for each of the following antioxidants:

(i) ascorbic acid (300 and 600 μM) (L-ascorbic acid product A4034; Sigma),
(ii) alpha tocopherol (30 and 60 μM) (TROLOX) (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carbonal product 23881.3; Aldrich, Gillingham, UK),
(iii) ascorbic acid (300 μM) + alpha tocopherol (30 μM) (TROLOX) and ascorbic acid (600 μM) + alpha tocopherol (60 μM) (TROLOX),
(iv) urate (200 and 400 μM),
(v) acetyl cysteine (5 and 10 μM) (N-acetyl cysteine product A8199; Sigma).

To determine at which stage of the protocol the antioxidants might be protecting the DNA, an additional 20 samples were each divided into three aliquots. One part was untreated; in the second part ascorbic acid (600 μM) was present in the Percoll preparation but not throughout the comet assay protocol; and the third part was prepared by Percoll without ascorbic acid, followed by the comet assay protocol in which ascorbic acid was present throughout (600 μM).

Induced damage

Each of the three aliquots of spermatozoa from the 150 subjects (the control and two treated with antioxidants) was divided into two further aliquots, so that spermatozoa from each of the three treatment groups could be additionally treated with a damaging agent. To induce oxidative DNA damage, spermatozoa from each sample were X-ray irradiated with a dose of 30 Gy at room temperature (Hughes *et al.*, 1996). X-ray irradiations were performed using a 300 kV Siemens Stabilipan X-ray source at a dose rate of 2.6 Gy min^{-1}. A set of six aliquots of spermatozoa from each subject was therefore obtained; (i) control spermatozoa, (ii) irradiated spermatozoa, (iii) spermatozoa treated with a low concentration of an antioxidant, (iv) spermatozoa treated with a low concentration of an antioxidant plus irradiation, (v) spermatozoa treated with a higher concentration of an antioxidant and (vi) spermatozoa treated with a higher concentration of an antioxidant plus irradiation.

Single cell gel electrophoresis assay

The modified alkaline comet assay for spermatozoa (Hughes *et al.*, 1996, 1997) was carried out on the prepared samples. For each sample, six slides were prepared, one for each of the aliquots treated as described above.

Fully frosted slides (Richards Supply Company Limited, London, UK) were covered with 100 μl of 0.5% normal melting point agarose (Sigma), a coverslip was added and the agarose was allowed to solidify. The coverslips were removed and 1×10^5 cells in 50 μl BWW were mixed with 50 μl of 1.2% low melting point agarose (Sigma) and used to form the second layer. The slides with coverslips removed were then placed in lysis buffer for 1 h [2.5 M NaCl, 100 mM NaEDTA, 10 mM Tris, 1% Triton X (Sigma) at a pH of 10]. The slides were then incubated at 37°C in 100 μl/ml of proteinase K (Sigma) in lysis buffer overnight. Antioxidants were present in the lysis buffer throughout the incubation.

After draining the proteinase K solution from the slides, they were placed in a horizontal electrophoresis unit filled with freshly prepared alkaline electrophoresis solution containing 300 mM NaOH and 1 mM EDTA (Sigma) for 20 min to allow the DNA to denature. Electrophoresis was performed at room temperature, at 25 V (0.714 V/cm) and 300 mA, obtained by adjusting the buffer level, for 10 min. The slides were then washed with a neutralizing solution of 0.4 M Tris (Sigma) at pH 7 to remove alkali and detergents. After neutralization, the slides were each stained with 50 μl of 20 μg/ml ethidium bromide (Sigma) and mounted with a coverslip. All steps were carried out under yellow light to prevent further DNA damage.

Analysis of cells and statistics

Fifty cells from each slide were selected randomly and analysed by image analysis using Hewlett and Packard Super VGA and Fenestra Komet Software (version 3). Observations were made at magnification ×400 using an epifluorescent microscope (Olympus BH2). Following preparation by the SCGE assay, each cell has the appearance of a 'comet' with a brightly fluorescent head and a 'tail' to one side, formed by the DNA which contains strand breaks being drawn away from the comet head into a tail during the electrophoresis (Figure 1). The DNA which remains in the 'head' of the comet, after specified electrophoresis times, gives a value for the amount of intact DNA

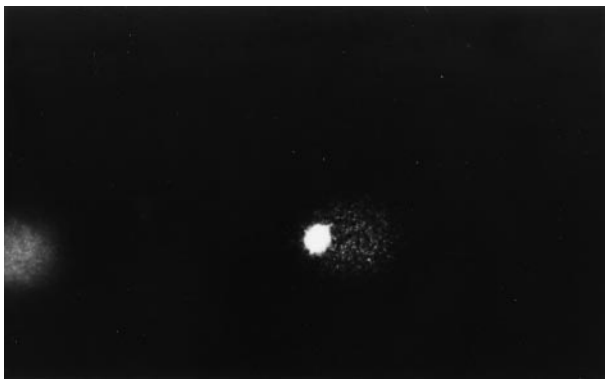

Figure 1. The typical appearance of a sperm 'comet' following preparation by the comet assay, showing the brightly fluorescent circular 'head' of the comet and the diffusely stained 'tail' of damaged DNA to one side of the head.

and is measured as 'percentage head DNA' by the software. Statistical analysis was carried out using non-parametric statistics with the Statistica (Statsoft Inc.) package on the values obtained for the percent head DNA of each cell.

The effect of sperm preparation technique on DNA integrity was assessed by the Mann–Whitney *U*-test. Analysis was carried out on the effect of the antioxidants on the sperm DNA using the Wilcoxon signed rank test by comparing the percentage head DNA values obtained for the control spermatozoa and those treated with an antioxidant. Further analysis using the Wilcoxon signed rank test was carried out to determine if the damage induced by the X-ray irradiation was significantly different from the control values within each sample and if the antioxidants had any protective effect from the induced oxidative damage.

Results

Comparison of sperm preparation techniques; Percoll and direct swim-up

The comparison of the two techniques showed that Percoll preparation isolated spermatozoa with significantly better DNA integrity ($P < 0.05$) than the direct swim-up technique. This was visualized by plotting the median value and interquartile range for the percentage head DNA readings obtained for the spermatozoa following each of the preparation techniques (Figure 2). There was no significant difference ($P > 0.05$) in DNA from spermatozoa isolated by swim-up with and without endotoxins.

Comparison of morphology and percent head DNA

Following Percoll preparation of the 150 samples used in the main study, there was no significant difference in percentage head DNA values between those samples for which the percentage normal morphology prior to Percoll was greater or less than 14% (Figure 3).

Stage of protocol influenced by antioxidants

Spermatozoa prepared by Percoll in the presence of ascorbic acid showed significantly higher percent head DNA values than both control spermatozoa ($P < 0.01$) and spermatozoa treated with ascorbic acid during the comet protocol only ($P < 0.05$). This can be observed by the distribution of percent

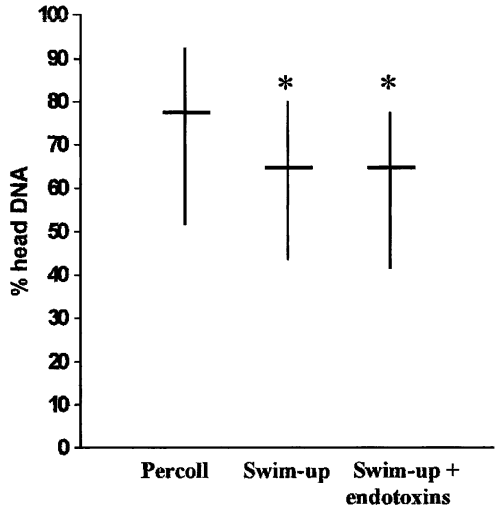

Figure 2. The median values and interquartile ranges for the % head DNA for spermatozoa obtained following preparation by either Percoll, direct swim-up or swim-up in which the BWW contained endotoxins. *Significantly different from Percoll preparation.

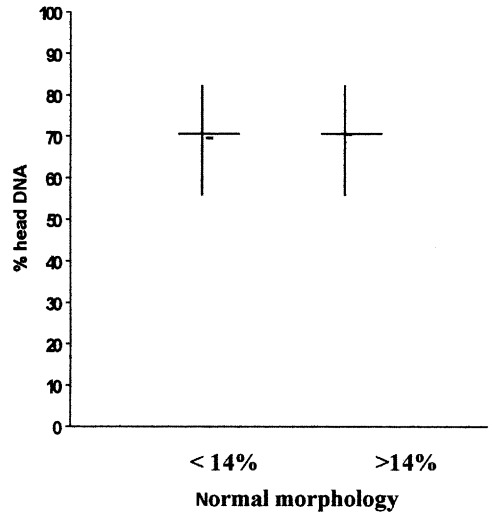

Figure 3. The median value and interquartile ranges for percent head DNA in spermatozoa from two groups divided according to percentage normal morphology before Percoll, i.e. those with normal morphology of greater or less than 14%.

head DNA (Figure 4), as a greater percentage of spermatozoa appear to fall into the higher percent head DNA ranges following Percoll preparation in the presence of the antioxidant in comparison to the control.

DNA integrity

Ascorbic acid supplementation

Sperm percentage head DNA (percent intact DNA) was significantly higher when ascorbic acid (600 μM) had been present during sperm preparation ($P < 0.001$), indicated by the increase in values determined for the median of each group (Table I). The values for the 1500 spermatozoa analysed (50 for each of the 30 subjects) were divided up into ranges of percentage head DNA, (0–10, 11–20, ... 91–100%), so that the distribution of DNA damage could be visualized. When this was carried

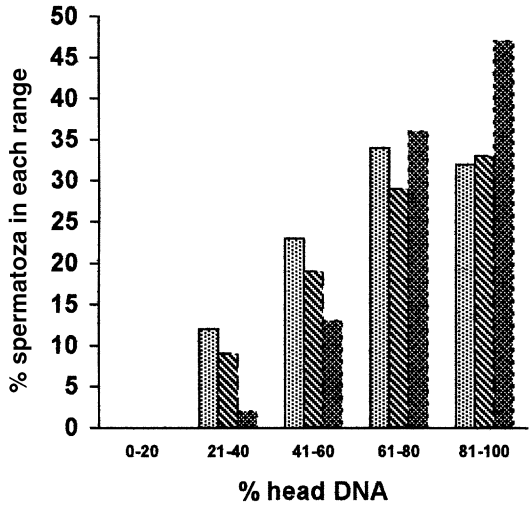

Figure 4. The DNA integrity of spermatozoa after Percoll preparation with no antioxidants (control) (▨), plus ascorbic acid (600 μM) present in the Percoll preparation (▩) or ascorbic acid (600 μM) added during the comet assay (▧). The graph shows the 1000 values obtained for each of the three treatments following division into ranges of percent head DNA, so that the percentage of spermatozoa within each range was plotted to give a distribution of DNA damage.

Table I. The effect of antioxidant present *in vitro* during Percoll preparation, X-ray irradiation and the comet assay protocol on sperm DNA. Values are percentage head DNA medians (interquartile range). Baseline values are those obtained from spermatozoa which were not treated by X-ray irradiation.

Antioxidant	Concentration	Baseline values	Irradiated (30 Gy) values
Ascorbic acid	Control	70.7 (80.6/54.8)	58.1 (78.2/41.1)††
	300 μM	71.1 (84.2/57.5)	64.1 (81.8/50.9)*,††
	600 μM	77.2 (87.8/57.5)**	73.0 (85.7/57.4)**,††
Alpha tocopherol	Control	71.5 (80.7/53.2)	61.5 (73.3/41.5)††
	30 μM	75.7 (87.0/56.1)**	64.8 (80.5/37.4)*,††
	60 μM	76.7 (86.9/52.3)**	65.4 (82.2/42.9)*,††
Urate	Control	70.8 (83.2/60.5)	61.2 (71.0/43.0)††
	200 μM	71.8 (85.7/62.6)	64.1 (74.4/45.6)*,††
	400 μM	75.9 (88.3/65.3)**	66.8 (74.8/47.4)*,††
Ascorbic acid + alpha tocopherol	Control	68.6 (88.5/48.7)	55.5 (80.2/39.5)††
	30 + 300 μM	57.2 (81.0/39.2)**	53.5 (802./35.8)
	60 + 600 μM	52.6 (77.5/34.5)**	50.5 (70.1/35.5)*,†
Acetyl cysteine	Control	71.1 (80.2/55.0)	57.7 (71.1/36.9)††
	5 μM	60.1 (70.1/46.6)**	49.6 (64.3/34.0)*,††
	10 μM	47.1 (63.3/35.5)**	42.5 (52.3/33.8)*,††

*Significantly different from control without antioxidant ($P < 0.01$).
**Significantly different from control without antioxidant ($P < 0.001$).
†Significantly different from spermatozoa without irradiation ($P < 0.01$).
††Significantly different from spermatozoa without irradiation ($P < 0.001$).

out for the control spermatozoa and those treated with the ascorbic acid, it was clear from these graphs that there was a shift in the distribution for percent head DNA towards a greater number of spermatozoa having more intact DNA following preparation in the presence of antioxidants (Figure 5A).

Following irradiation (30 Gy), there was a significant decrease in percent head DNA of the sperm population ($P < 0.001$), also indicated by the decrease in the median value for the population (Table I). In the presence of ascorbic acid (300 μM) and subsequent irradiation, the median percent head DNA value did not decrease to the same extent, and there was a significant increase in percent head DNA values for the population from the irradiated control values ($P < 0.01$). In fact, when spermatozoa were irradiated in the presence of ascorbic acid (600 μM), the resulting median percent head DNA value was similar to the median baseline control value ($P > 0.05$) (Table I).

Alpha tocopherol

In the presence of alpha tocopherol (30 or 60 μM), percent head DNA was significantly higher than the control values ($P < 0.001$), shown also as an increase in the median value for percent head DNA from that obtained for the control (Table I). This protective effect was also evident from plotting the distribution graph which shows that a greater number of spermatozoa with more intact DNA are obtained following Percoll in the presence of the antioxidant (Figure 5B).

Irradiation produced a significant decrease in percent head DNA which was lessened by the presence of alpha tocopherol (30 or 60 μM) (Table I). In the presence of the antioxidants, there was a significant increase in percent head DNA from the irradiated control ($P < 0.01$), as demonstrated by an increase in the median value for the population of spermatozoa in the presence of alpha tocopherol (Table I).

Urate

In the presence of urate (400 μM), there was a significant increase in the percent head DNA over the control values ($P < 0.001$), along with an increase in the median value (Table I). There was also a shift to the right in the distribution graph (Figure 5C), again showing a greater number of spermatozoa with more intact DNA in the presence of urate.

The antioxidant also provided protection from induced DNA damage as the percent head DNA in the presence of urate (200 or 400 μM) was significantly improved from the irradiated control level ($P < 0.01$). Again, this can be observed as an increase in the median value for the population from the irradiated control in the presence of urate (Table I).

Ascorbic acid + alpha tocopherol

The presence of ascorbic acid and tocopherol together throughout the protocol (Table I, Figure 5D) caused a decrease in percent head DNA ($P < 0.001$), indicating that DNA damage had been induced. These antioxidant supplementations also resulted in significant DNA damage additional to that induced by the irradiation shown in Table I by the decrease in the median value in the presence of antioxidants from the irradiated control.

Acetyl cysteine

The presence of acetyl cysteine (Figure 5E) also induced significant DNA damage with a decrease in percent head DNA for the sperm population ($P < 0.001$) and a decrease in the median value (Table I). Following irradiation further DNA damage was caused in addition to that induced by the irradiation (Table I).

Discussion

The most important sperm parameter for assisted conception is intact DNA, especially for the techniques which select

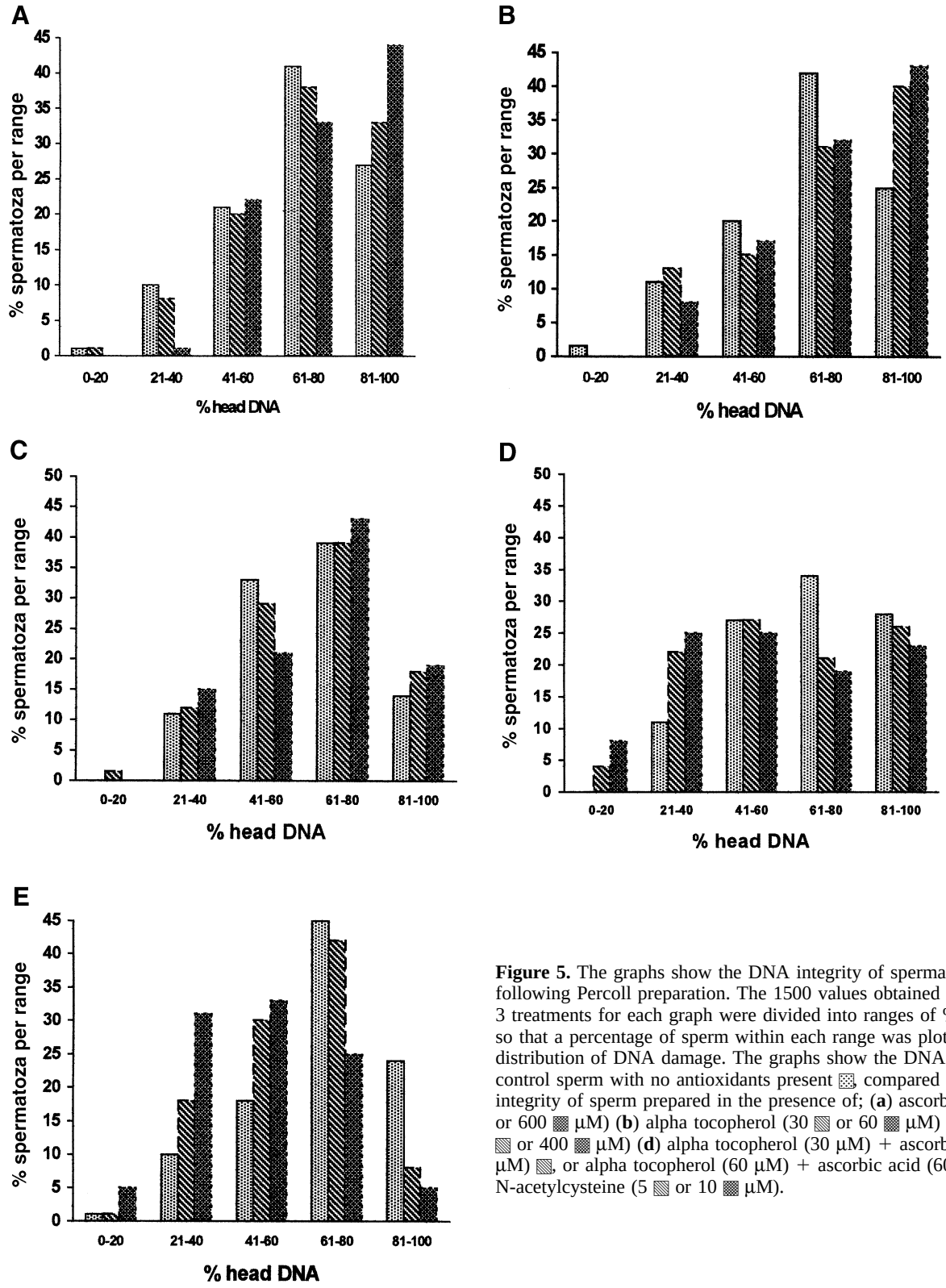

Figure 5. The graphs show the DNA integrity of spermatozoa obtained following Percoll preparation. The 1500 values obtained for each of the 3 treatments for each graph were divided into ranges of % head DNA, so that a percentage of sperm within each range was plotted to give a distribution of DNA damage. The graphs show the DNA integrity of control sperm with no antioxidants present ⬚, compared to the DNA integrity of sperm prepared in the presence of; (**a**) ascorbic acid (300 ▧ or 600 ▩ μM) (**b**) alpha tocopherol (30 ▧ or 60 ▩ μM) (**c**) urate (200 ▧ or 400 ▩ μM) (**d**) alpha tocopherol (30 μM) + ascorbic acid (300 μM) ▧, or alpha tocopherol (60 μM) + ascorbic acid (600 μM) ▩ (**e**) N-acetylcysteine (5 ▧ or 10 ▩ μM).

few spermatozoa for fertilization purposes, such as subzonal insemination (SUZI), or indeed only one spermatozoon, as is the case with ICSI. Although DNA integrity has not been correlated with in-vitro fertilization (IVF) rates, probably due to the competition between the large number of spermatozoa used, it has been directly correlated with SUZI, as defective DNA packaging due to lack of protamines resulted in reduced fertilization rates (Bianchi *et al.*, 1993). Damage to this DNA may be due to the increased generation of reactive oxygen species that, under normal conditions, is limited to the low steady generation of superoxide and hydrogen peroxide required for capacitation (De Lamirande *et al.*, 1997). However, there is a potential for increased generation of ROS from defective spermatozoa, the mechanism of which is unclear but

which may involve increased availability of NADPH from excess cytoplasm, or electron leakage from damaged mitochondria (Aitken, 1997).

Spermatozoa have only two defence mechanisms against oxidative attack of their DNA; the packaging arrangement of the DNA, and the seminal plasma. During spermatogenesis, the chromatin becomes highly condensed within a protamine matrix (Sidney *et al.*, 1986). The DNA is organized into loops, attached at their bases to the nuclear matrix, anchored to the base of the sperm tail by the nuclear annulus and stabilized by disulphide bonds (Ward, 1993; Barone *et al.*, 1994). This tight packing of the DNA reduces exposure to free radical attack. The second line of defence is the antioxidant capacity of its seminal plasma (Lewis *et al.*, 1995; 1997).

Sperm preparation techniques are used routinely in assisted conception units to isolate the spermatozoa with the best motility and morphology from seminal plasma, damaged spermatozoa and other debris present in the semen. Although studies with acridine orange have shown that both Percoll and swim-up procedures may select spermatozoa with more mature nuclei (Golan *et al.*, 1997), here we have shown that Percoll gradients concentrate spermatozoa with more intact DNA than the swim-up process (Figure 2) which is in agreement with other studies (Le Lannou and Blanchard, 1988; Colleu *et al.*, 1996). As direct swim-up depends only on the swimming properties of the spermatozoa, those with defective nuclei may also swim-up into the top layer of BWW. In contrast, the Percoll technique also relies on the density of the spermatozoa. Since an intact nucleus is tightly packed (Ward, 1993; Barone *et al.*, 1994) and therefore more dense, it is predictable that these should be concentrated in the bottom layer of Percoll (Pasteur *et al.*, 1991).

In the present study, the percentage normal morphology before Percoll preparation was not related to the DNA integrity after Percoll (Figure 3). This is in agreement with Bianchi *et al.* (1993), who showed that morphologically normal spermatozoa may possess loosely packed defective chromatin. On the other hand, Hall *et al.* (1995) showed that Percoll centrifugation decreases the percentage of spermatozoa with head abnormalities which may also reflect the lack of relationship between the pre-Percoll morphology and DNA packaging, as the ratio of normal morphology may change pre- and post-Percoll. Therefore, it would be of interest to determine any relationship between the post-Percoll morphology and sperm DNA integrity.

Percoll has recently been removed from the market for use in assisted conception due to the presence of 'uncontrolled substances' (Pharmacia Biotech, formal notification, 1997). This study has shown that, in terms of DNA quality, Percoll appears to have no detrimental effects and that endotoxins present at these concentrations are of no consequence (Figure 2).

Baseline DNA integrity in spermatozoa is lower than that of somatic cell types (Hughes *et al.*, 1996), possibly a reflection of its physiological role (Singh *et al.*, 1989). Human sperm samples were also found to exhibit a wide variation in DNA damage as measured by the comet assay (Hughes *et al.*, 1996), which is in agreement with the variation found in CMA_3 fluorochrome staining measuring the protamine content of the nucleus, and the amount of endogenous DNA nicks measured by in-situ nick translation (Bianchi *et al.*, 1993; Sakkas *et al.*, 1995). Spontaneous lipid peroxidation in human spermatozoa occurs at highly variable rates in different sperm samples, and is largely dependent on the intracellular content of superoxide dismutase which varies between spermatozoa (Storey *et al.*, 1997). This variability in antioxidant protection may explain the variation in DNA damage found in individual spermatozoa within one sample. The present study suggests that some of the DNA damage found in a sperm population may be due to the removal of the seminal plasma during preparation for IVF, as this procedure also removes the spermatozoa from their main source of antioxidant protection. Addition of various chain-breaking antioxidants to the media resulted in protecting the DNA from subsequent damage.

Ascorbic acid is the major contributor to the chain-breaking antioxidant capacity of seminal plasma (Lewis *et al.*, 1997) the concentration of which is 10 times higher than that of blood plasma and is actively secreted by the seminal vesicles during ejaculation (Berg *et al.*, 1941). When ascorbic acid was added to the media at concentrations found by Lewis *et al.* (1997) in seminal plasma of fertile men, it protected spermatozoa from DNA damage *in vitro*. This protection was previously shown *in vivo* (Fraga *et al.*, 1991; Jacob *et al.*, 1991), by measuring the oxidized nucleoside 8-hydroxy-2′-dehydroxyguanosine (oxo8dG), one of the major products of DNA damage. Ascorbic acid has been shown to scavenge most ROS (Sies *et al.*, 1992) which undoubtedly explains its efficiency.

Alpha tocopherol has been shown to inhibit sperm lipid peroxidation *in vitro* (Aitken and Clarkson, 1988), to enhance the ability of spermatozoa to fuse with zona-free hamster oocytes (Aitken *et al.*, 1989) and improve zona pellucida binding (Kessopoulou *et al.*, 1995). In this study, we found alpha tocopherol (30 μM) provided protection to DNA, which is in agreement with protection afforded *in vitro* by this antioxidant (Fraga *et al.*, 1996), making it a multifunctional antioxidant and a potentially useful supplement to sperm preparation media.

Although ascorbic acid and alpha tocopherol supplemented separately produced protective effects from DNA damage, when added together they produced a damaging effect. It is known that in high concentrations, ascorbic acid can be potentially oxidative (Gutteridge, 1994). However, the ascorbic acid concentrations used with alpha tocopherol were the same as those used separately. The trace tocopherol levels found by Lewis *et al.* (1997) may be sufficient to combat oxidative assault when present with ascorbic acid due to its regeneration (Kagan, 1992), so the higher concentration used in this study may have caused an abnormal redox balance. Similar results were found by Sweetman *et al.* (1997), who found a protective effect from ascorbic acid or tocopherol individually, but not together, on human lymphoblast cells. It was suggested that there is a narrow physiological range in which these antioxidants can work synergistically. Similar results were observed in our laboratory in a pilot study *in vivo* (results not shown). Supplementation with ascorbic acid (350 mg) and alpha tocopherol (250 mg) together for 1 month induced substantial damage to the sperm DNA, whereas supplementation with the

antioxidants individually had no damaging effect and in some cases enhanced DNA integrity.

The other major chain-breaking antioxidant in seminal plasma is urate (Lewis *et al.*, 1997). Seminal plasma concentrations of urates were found to decrease in normozoospermic infertile men (Thiele *et al.*, 1995), although this was not observed by Lewis *et al.* (1997). Urate binds transition metals, thus preventing Fe^{2+}-driven free radical reactions, and also scavenges peroxyl and hydroxyl radicals (Sevanian *et al.*, 1991). Here, urate at the higher concentration of 400 μM prevented damage to the sperm DNA, suggesting that an optimal concentration of urate is required for antioxidant protection.

N-Acetyl cysteine (NAC) can act as an antioxidant through its role as a precursor of glutathione (GSH) synthesis and at an extracellular level, where it acts directly on oxidants (De Vries and De Flora, 1993). The antioxidant has been shown to be beneficial on sperm motility *in vitro* (Baker *et al.*, 1996) and sperm DNA (Den Boer, 1990). In contrast, in the present study the effect of acetyl cysteine was to damage the DNA. One explanation for the damaging effect of the NAC may be that in the spermatids thiols act to preserve the S–H groups in the reduced state by disulphide exchange (Knox, 1960; Evenson *et al.*, 1993), whereas in ejaculated spermatozoa the DNA becomes stabilized by S–S bonds in the presence of zinc in the seminal fluid (Molina *et al.*, 1995). Perhaps the presence of acetyl cysteine in the absence of zinc in the Percoll medium has disturbed the thiol:disulphide ratio, reducing the S–S bonds and so destabilizing the sperm DNA allowing damage to occur.

While substantial quantities of glutathione are found in the testis, reproductive tract fluid and epididymal spermatozoa, much less is present in ejaculated spermatozoa (Agrawal and Vanha-Perttula, 1988). This may be a consequence of the small cytoplasmic content of mature spermatozoa which would in turn reduce the amount of enzymes available for the formation of glutathione. This suggests that the effect of NAC on spermatozoa comes not from its role as a precursor of glutathione but through its extracellular role. Therefore an alternative explanation for the DNA damage observed in the present study is the direct extracellular action of NAC. Under these circumstances, NAC may initiate lipid peroxidation and so may be acting as a pro-oxidant. This is supported by the fact that levels of thiols in general were found to be higher in infertile men (Lewis *et al.*, 1997), who also demonstrated an increase in ROS activity.

Spermatozoa produced by infertile men were shown to have DNA which was more susceptible to damage by irradiation than that from fertile men (Hughes *et al.*, 1996). X-ray irradiation, the source of damaging agent used in the present study, acts by ionizing water to produce hydroxyl radicals (Hall, 1994) and by producing single and double strand breaks directly. Ascorbic acid efficiently scavenges these radicals (Sies *et al.*, 1992) and its presence in this study prevented induced damage from the X-ray irradiation (Table I). As a scavenger of hydroxyl radicals, urate fulfils a similar role to that observed with ascorbic acid. Although tocopherol prevented induced damage to some extent, the DNA integrity did not return to baseline as observed with ascorbic acid. Tocopherol is found in the cell membrane, in contrast to ascorbic acid which, being water soluble, is found in the cell cytoplasm where the hydroxyl radicals are formed, leaving it closer to the oxidative attack. Previous reports have indicated that where ascorbic acid plays a dominant role in protection from irradiation, alpha tocopherol is more important in protection against chemicals which must cross the cell membrane to gain access to the cell (Sweertman *et al.*, 1997).

We have shown in this study that DNA can be protected during sperm Percoll centrifugation preparation by the presence of antioxidants in the media. Ascorbic acid, alpha tocopherol and urate separately significantly increased the percentage of spermatozoa with good DNA integrity. The antioxidants also protected the spermatozoa from induced in-vitro oxidative DNA damage by irradiation. Ascorbic acid and urate, being water soluble hydroxyl scavengers and found in the highest concentrations in seminal plasma, are the most effective antioxidants for this role.

References

Agrawal, Y.P. and Vahna-Perttula, T. (1988) Glutathione, L-glutamic acid and gamma-glutamyl transpeptidase in the bull reproductive tissues. *Int. J. Androl.*, **11,** 123–131.

Aitken, R.J. (1997) Molecular mechanisms regulating sperm function. *Mol. Hum. Reprod.*, **3,** 169–173.

Aitken, R.J. and Clarkson, J.S. (1987) Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. *J. Reprod. Fert.*, **81,** 459–469.

Aitken, R.J. and Clarkson, J.S. (1988) Significance of reactive oxygen species and antioxidants in defining the efficiency of sperm preparation techniques. *J. Androl.*, **9,** 367–376.

Aitken, R.J. and West, K.M. (1990) Analysis of the relationship between reactive oxygen species production and leucocyte infiltration in fractions of human semen separated by Percoll gradients. *Int. J. Androl.*, **3,** 433–451.

Aitken, R.J., Clarkson, J.S. and Fisher, S. (1989) Generation of reactive oxygen species, lipid peroxidation and human sperm function. *Biol. Reprod.*, **40,** 183–197.

Aitken, R.J., Buckingham, D., West, K. *et al.* (1992) On the contribution of leukocytes and spermatozoa to the high levels of reactive oxygen species recorded in the ejaculates of oligozoospermic patients. *J. Reprod. Fert.*, **94,** 451–462.

Alvarez, J.G., Touchstone, J.C., Blasc, L. and Storey, B.T. (1987) Spontaneous lipid peroxidation and production of hydrogen peroxide and superoxide in human spermatozoa. *J. Androl.*, **8,** 338–345.

Baker, H.W.G. (1993) Treatment of male infertility – what does the future hold? *Int. J. Androl.*, **16,** 343–348.

Baker, H.W., Brindle, J.B., Irvine, D.S. and Aitken, R.J. (1996) Protective effect of antioxidants on the impairment of sperm motility by activated polymorphonuclear leucocytes. *Fertil. Steril.*, **65,** 411–419.

Barone, J.G., De Lara, J., Cummings, K.B. and Ward, W.S. (1994) DNA organisation in human spermatozoa. *J. Androl.*, **15,** 139–144.

Berg, M.B., Huggins, C. and Hodges, C.V. (1941) Concentration of ascorbic acid and the phosphatase in secretion of the male genital tract. *Am. J. Physiol.*, **133,** 82–87.

Bianchi, P.G., Manicardi, G.C., Bizzaro, D. *et al.* (1993) Effect of DNA protamination on fluorochrome staining and *in situ* nick translation of murine and human sperm. *Biol. Reprod.*, **49,** 1083–1088.

Biggers, J.D., Whitten, W.K. and Whittingham, D.G. (1971) The culture of mouse embryos *in vitro*. In David, J.C. (ed.), *Methods in Mammalian Embryology.* Freeman Press, San Francisco, pp. 86–94.

Chandley, A.C. and Kofman-Algaro, S. (1971) Unscheduled DNA synthesis in human germ cells following UV irradiation. *Exp. Cell Res.*, **69,** 45–48.

Colleu, D., Lescoat, D. and Gouranton, J. (1996) Nuclear maturity of human spermatozoa selected by swim-up or by Percoll gradient centrifugation procedures. **65,** 160–164.

De Lamirande, E., Leclerc, P. and Gagnon, C. (1997) Capacitation as a

regulatory event that primes spermatozoa for the acrosome reaction and fertilisation. *Mol. Hum. Reprod.*, **3,** 175–193.

Den Boer, P.J., van Loon, A.A.W.M., Mackenbach, P. *et al.* (1990) Effect of glutathione depletion on the cytotoxicity of xenobiotics and induction of single strand DNA breaks by ionizing radiation in isolated hamster round spermatids. *J. Reprod. Fert.*, **88,** 159–169.

De Vries, N. and De Flora, S. (1993) *N*-Acetyl-L-cysteine. *J. Cell. Biochem. Supp.*, **17,** 270–277.

Evenson, D., Jost, L.K. and Gandy, J. (1993) Glutathione depletion potentiates ethyl methansulfonate-induced damage to sperm chromatin structure. *Reprod. Toxicol.*, **7,** 197–304.

Fleming, S., Green, S., Hall, J. *et al.* (1995) Analysis and alleviation of male infertility. *Microsc. Anal.*, 35–37.

Fraga, C.G., Motchnik, P.A., Shigenaga, M.K. *et al.* (1991) Ascorbic acid protects against endogenous oxidative damage in human sperm. *Proc. Natl. Acad. Sci.*, **88,** 11003–11006.

Fraga, C.G., Motchnik, P.A., Wyrobek, A.J. *et al.* (1996) Smoking and low antioxidant levels increase oxidative damage to sperm DNA. *Mutat. Res.*, **351,** 199–203.

Golan, R., Shochat, L., Weissenberg, R. *et al.* (1997) Evaluation of chromatin decondensation in human spermatozoa: a flow cytometric assay using acridine orange staining. *Mol. Hum. Reprod.*, **3,** 47–54.

Gutteridge, J.M.C. (1994) Antioxidants, nutritional supplements and life threatening diseases. *Br. J. Biomed. Sci.*, **51,** 288–195.

Hall, J.A., Fishel, S.B., Timson, J.A. *et al.* (1995) Human sperm morphology evaluation pre- and post-Percoll gradient centrifugation. *Hum. Reprod.*, **10,** 342–346.

Hughes, C.M., Lewis, S.E.M., McKelvey-Martin, V.J. and Thompson, W. (1996) A comparison of baseline and induced DNA damage in human sperm from fertile and infertile man, using a modified comet assay. *Mol. Hum. Reprod.*, **2,** 613–619.

Hughes, C.M., Lewis, S.E.M., McKelvey-Martin, V.J. and Thompson, W. (1997) Reproducibility of human sperm DNA measurements using the single cell gel electrophoresis assay. *Mutat. Res.*, **347,** 261–268.

Jacob, R.A., Kelley, D.S., Pianalto, F.S. *et al.* (1991) Immunocompetence and oxidant defense during ascorbate depletion of healthy men. *Am. J. Clin. Nutr.*, **54,** 1302–1309.

Jones, R., Mann, T. and Sherrins, R.J. (1973) Adverse effects of peroxidized lipid on human spermatozoa. *Proc. R. Soc. Lond. (Biol.)*, **201,** 413–417.

Kagan, V.E., Serbinova, E.A., Forte, T. *et al.* (1992) Recycling of vitamin E in low density lipoproteins. *J. Lipid Res.*, **33,** 385–397.

Kessopoulou, E., Tomlinson, M.J., Barratt, C.L.R. *et al.* (1992) Origin of reactive oxygen species in human semen: spermatozoa or leukocytes? *J. Reprod. Fertil.*, **94,** 463–470.

Kessopoulou, E., Powers, H.J., Sharma, K.K. *et al.* (1995) A double blind randomized placebo cross-over controlled trial using the antioxidant vitamin E to treat reactive oxygen species associated with male infertility. *Fertil. Steril.*, **64,** 825–831.

Knox, W.E. (1960) Glutathione. In Boyer, P.D., Lardy, H. and Myrback, K. (eds), *The Enzymes*, 2nd edn, vol. 2. Academic Press, New York, pp. 253–294.

Kruger, T.F., Ackerman, S.B., Simmons, K.F. *et al.* (1987) A quick reliable staining technique for human sperm morphology. *Arch. Androl.*, **18,** 275–277.

Le Lannou, D. and Blanchard, Y. (1988) Nuclear maturity and morphology of human spermatozoa selected by Percoll density gradient centrifugation or by swim-up procedure. **84**, 551-556.

Lewis, S.E.M., Boyle, P.M., McKinney, K.A. *et al.* (1995) Total antioxidant capacity of seminal plasma is different in fertile and infertile men. *Fertil. Steril.*, **64,** 866–870.

Lewis, S.E.M., Sterling, S. Young, I. and Thompson, W. (1997) Comparison of individual antioxidants and total antioxidant capacity of sperm and seminal plasma in fertile and infertile men. *Fertil. Steril.*, **67,** 142–147.

Liu, D.Y. and Baker, H.W.G. (1988) The proportion of sperm with poor morphology but normal intact acrosomes detected with pisum sativum agglutin correlates with fertilisation *in vitro*. *Fertil. Steril.*, **50,** 288–293.

Liu, D., Lopata, A., Johnson, W.I.H. and Baker, H.W.G. (1988) A human sperm zona pellucida binding test using oocytes that failed to fertilise *in vitro*. *Fertil. Steril.*, **50,** 782–788.

McKelvey-Martin, V.J., Green, M.H.L., Schmer, P. *et al.* (1993) The single cell gel electrophoresis assay (comet assay): a European review. *Mutat. Res.*, **288,** 47–63.

Molina, J., Castilla, J.A., Gil, T. *et al.* (1995) Influence of incubation on the chromatin condensation and nuclear stability of human spermatozoa by flow cytometry. *Mol. Hum. Reprod.*, **10,** 1280–1286.

Palmero, G., Joris H., Devroey, P. and Van Steirteghem, A. (1992) Pregnancies after intracytoplasmic sperm injection of single spermatozoon into an oocyte. *Lancet*, **340,** 17.

Pasteur, X., Maubon, I., Sabido, O. *et al.* (1991) Comparison of the chromatin stainability of human spermatozoa separated by discontinuous Percoll gradient centrifugation. *Anal. Cytol. Histol.*, **14,** 96–104.

Sakkas, D., Manicardi, G.C., Bianchi, P.G. *et al.* (1995) Relationship between the presence of endogenous nicks and sperm chromatin packaging in maturing and fertilising mouse sperm. *Biol. Reprod.*, **52,** 1149–1155

Sevanian, A., Davies, K.J.A. and Hochstein, P. (1991) Serum urate as an antioxidant for ascorbic acid. *Am. J. Clin. Nutr.*, **54,** 1129–1134.

Sidney, R. and Grimes, J.R. (1986) Nuclear proteins in spermatogenesis. *Comp. Biochem. Physiol.*, **83,** 495–500.

Sies, H., Stahl, W. and Sundquist, A.R. (1992) Antioxidant function of vitamins. *Ann. N. Y. Acad. Sci.*, **669,** 7–20.

Singh, N.P., Banner, D., Tice, R.R. *et al.* (1989) Abundant alkali sensitive sites in DNA of human and mouse sperm. *Exp. Cell Res.*, **184,** 461–470.

Steenken, S. (1989) Purine bases, nucleosides and nucleotides: aqueous solution redox chemistry and transformation reactions of their radical cations and e- and OH adducts. *Chem. Rev.*, **89,** 503–520.

Storey, B.T. (1997) Biochemistry of the induction and prevention of lipoperoxidative damage in human sperm. *Mol. Hum. Reprod.*, **3,** 203–213.

Sweetman, S.F., Strain, J.J and McKelvey-Martin, V.J. (1997) Effect of antioxidant vitamin supplementation on DNA damage and repair in human lymphoblastoid cells. *Nutr. Cancer*, **27,** 122–130.

Thiele, J.J., Freisleben, H.J., Fuchs, J. and Oschsendorf, F.R. (1995) Ascorbic acid and urate in human seminal plasma: determination and interrelationships with chemiluminescence in washed semen. *Hum. Reprod.*, **10,** 110–115.

Van Loon, A.A.W.M., Den Boer, P.J., Van der Schans, G.P. *et al.* (1991) Immunochemical detection of DNA damage induction and repair at different cellular stages of spermatogenesis of the hamster after *in vitro* or *in vivo* exposure to ionising radiation. *Exp. Cell Res.*, **193,** 303–309.

Van Steirteghem, A., Liu, J., Joris, H. *et al.* (1994) High fertilisation and implantation rates after intracytoplasmic sperm injection. *Hum. Reprod.*, **8,** 1061–1066.

Ward, W.S. (1993) DNA loop domain tertiary structure in mammalian spermatozoa. *Biol. Reprod.*, **48,** 1193–1201.

Ward, W.W. and Coffey, D.S. (1991) DNA packaging and organisation in mammalian spermatogenesis: comparison with somatic cells. *Biol. Reprod.*, **44,** 569–574.

World Health Organization (1992) *Laboratory Manual for the Examination of Semen and Sperm–Cervical Mucus Interaction*. Cambridge University Press, Cambridge, p. 107.

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