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DNA Integrity in Human Spermatozoa: Relationships With Semen Quality

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ABSTRACT: The literature contains conflicting evidence regarding the existence of DNA damage in spermatozoa from infertile male patients. To examine this phenomenon, we have studied ejaculated spermatozoa from normozoospermic semen donors and from a group of the unselected male partners of couples attending an infertility clinic for initial investigation. Classical semen analysis according to World Health Organization (WHO) guidelines was undertaken with computer-assisted sperm analysis (CASA). Spermatozoa were prepared by sequential washing and centrifugation and were analyzed for DNA fragmentation using three assays: 1) a single-cell gel electrophoresis (comet) assay, 2) in situ nick translation with prior chemical decondensation (ISNT-decondensed), and 3) in situ nick translation without prior chemical decondensation (ISNT-condensed). In addition, reactive oxygen species (ROS) generation by spermatozoa was measured, and seminal plasma was analyzed for its total reactive antioxidant potential (TRAP). When the donor and patient groups were compared, the latter had lower levels of semen quality and higher levels of DNA damage, which was particularly apparent using the comet assay. Highly significant negative correlations were observed between DNA fragmentation, detected by all three assays, and semen quality, particularly sperm concentration. In addition, multiple regression analysis indicated that other attributes of semen quality, such as sperm movement and ROS generation, were also related to DNA damage. We conclude that a significant proportion of infertile men have elevated levels of DNA damage in their ejaculated spermatozoa.

Key words: Comet assay, in situ nick translation, infertility, spermatogenesis, ICSI, CASA.

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Infertility remains a major clinical problem, with recent data from Europe suggesting that as many as one in four couples trying to conceive now experience difficulties (Schmidt et al, 1995). Moreover, in this and other studies, the most prevalent causative factor appears to be defective semen quality (Thonneau et al, 1991; Hull, 1992; Templeton, 1992). While there can be no doubt that the recent introduction of microassisted fertilization techniques has revolutionized the management of couples with male-factor infertility (Van Steirteghem et al, 1993a,b), concerns over the safety of this approach remain (Cummins and Jequier, 1995; Foresta et al, 1996). Painstaking follow-up studies of children born after microassisted fertilization have generated broadly reassuring data, with no evidence of an increase in the incidence of major congenital malformations amongst children born after intracytoplasmic sperm injection (ICSI; Bonduelle et al, 1996a,b; Wisanto et al, 1996). This view has recently been challenged, however (Kurinczuk and Bower, 1997).

An additional concern arises from our understanding of the etiology of defective sperm function in couples with male infertility. One feature of the semen of infertile men, particularly those with oligoasthenozoospermia, is the production of excessive levels of reactive oxygen species (ROS) (Aitken et al, 1989a,b, 1991). It has been shown that these excessive ROS can emanate from contaminating leukocytes (Aitken et al, 1994, 1995) or from dysfunctional spermatozoa (Aitken et al, 1992; Iwasaki and Gagnon, 1992; Gomez et al, 1996). One consequence of excessive ROS generation is peroxidative damage to the plasma membrane, which leads to an impairment of sperm function that is reflected in decreased pregnancy rates in vivo as well as in impaired fertilization in vitro (Aitken et al, 1989b; 1991; Sukcharoen et al, 1996). While it is known that ROS have the capacity to damage lipids in the sperm plasma membrane, it is also known that ROS can damage other cellular structures, particularly DNA (Dizdaroglu, 1992; Altman et al, 1995; Palomba et al, 1996; Lloyd et al, 1997). In the context of spontaneous pregnancy or conception following in vitro fertilization (IVF), spermatozoa exposed to ROS become dysfunctional as a consequence of this peroxidative damage to the plasma membrane and are unable to initiate fertilization. Under these circumstances, any genetic damage induced by exposure of spermatozoa to oxidative stress is unlikely to be transmitted to the embryo. In the context of ICSI, however, these safeguards are removed, and there is nothing to prevent spermatozoa with dam-

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aged DNA being injected directly into the oocyte. Because DNA damage appears to have no impact upon the ability of the spermatozoon to achieve fertilization following ICSI (Twigg et al, 1997), the probability that the procedure might result in the transmission of genetic damage to the embryo cannot be discounted. In a recent study, we have exposed spermatozoa from normozoospermic men to oxidizing environments created by coincubation with hydrogen peroxide, reduced nicotinamide adenine dinucleotide phosphate (NADPH), or activated white cells. The subsequent ability of the spermatozoa to decondense in vitro was examined using sequential incubations in EDTA, dithiothreitol, and sodium dodecyl sulphate, and the amount of DNA strand breakage were assessed by an in situ nick translation (ISNT) protocol. Finally, cells exposed to hydrogen peroxide, NADPH, and activated leukocytes were microinjected into hamster oocytes, and their ability to decondense and form normal pronuclei was determined. The results indicated that human sperm chromatin becomes cross-linked under conditions of oxidative stress and exhibits increased DNA strand breakage, yet the rate of pronucleus formation was no different from that of untreated control cells. At present, very little is known of the level of DNA damage encountered in human spermatozoa or the relationship between such damage and other attributes of semen quality. Previous work has established relationships between ROS generation and impaired sperm maturity, measured by cytoplasmic retention and other measures (Huszar and Vigue, 1994; Gomez et al, 1996; Lalwani et al, 1996), and it is possible that markers of sperm maturity such as G6PDH, creatine phosphokinase, and lactate dehydrogenase-x may modulate the relationship between semen quality and the ROS-mediated decline of DNA integrity.

A number of approaches to the assessment of DNA damage have been described, including nick-end labeling (Baccetti et al, 1996), ISNT (Sakkas et al, 1995), single-cell gel electrophoresis or the comet assay (McKelvey-Martin et al, 1993), and measurement by high-pressure liquid chromatography of oxidized DNA (Fraga et al, 1996). Application of these assays to human spermatozoa has generated conflicting results. Thus, Sun and colleagues (Sun et al, 1997), using the nick-end labeling or TUNEL assay in a study of unselected men undergoing in vitro fertilization treatment, have suggested that a negative relationship exists between the observed extent of DNA damage in human sperm and the conventional parameters of semen quality. In contrast, Hughes and coworkers (Hughes et al, 1996), using the comet assay, found no difference in the extent of underlying DNA damage when infertile men were compared with normal controls. Previous work with other cell types has tended to suggest that the comet assay is the most sensitive (Leroy et al, 1996).

Because of this discrepancy in the literature and because of the potential clinical importance of sperm DNA damage in the context of assisted reproduction, the present study was undertaken to evaluate DNA fragmentation in the spermatozoa of both normozoospermic donors and infertile men attending an infertility clinic as part of their routine investigation. A range of techniques has been used to assess DNA damage, the extent of which has then been correlated with various attributes of semen quality. It should be noted that the conventional criteria for semen quality (World Health Organization [WHO], 1992) represent measurements made on populations of cells, whereas the assays used to quantify DNA integrity are normally at the level of the single cell.

Materials and Methods

This study was approved by the local ethical committee, and all patients and volunteers gave written informed consent to the use of their gametes for research study. The laboratory that conducted the semen analysis was participating in one national and one international externally assessed quality control scheme throughout the duration of this study.

Subjects and Sample Collection

Semen samples were collected from an unselected group of men attending the Infertility Clinic of the Royal Infirmary of Edinburgh for the investigation of infertility. Normozoospermic volunteer donors participating in our research program and who had been carefully screened for the absence of significant reproductive disease provided control samples. In all cases, after 3–4 days of sexual abstinence, samples were collected by masturbation into wide-mouthed sterile containers and were delivered to the laboratory within 1 hour of ejaculation.

Assessment of the Conventional Criteria of Semen Quality

Semen samples were allowed to liquefy at 37°C, following which an aliquot was removed in order to construct a conventional semen profile composed of ejaculate volume (in milliliters), sperm concentration ($\times 10^6$ per milliliter), overall and progressive motility (percentage), and normal morphology (percentage) using the guidelines promulgated by the WHO (1992). Duplicate assessment of sperm concentration was made by using a positive-displacement pipette to add 10 μL of semen to 190 μL of sperm-diluting fluid (SDF; 25g $NaHCO_3$ in 5 mL w/v 40% formalin, made up to 500 mL with distilled H_2O), following which the preparation was thoroughly mixed and loaded onto a hemocytometer (Improved Neubauer; Weber Scientific Instruments, Sussex, United Kingdom). The loaded chamber was allowed to settle for 5–15 minutes in a humidified chamber before counting at ×10 magnification. For the assessment of motility, 10 μL of semen was placed on a microscope slide prewarmed to 37°C and was covered with a 19-mm × 19-mm coverslip. The preparation was then examined at 37°C at ×100 magnification with phase–contrast optics with the aid of a grid on an eyepiece

graticule. Spermatozoa in WHO categories a, b, and c were considered motile, and those in categories a and b were considered progressively motile.

For the assessment of sperm morphology, a positive-displacement pipette was used to take 50 μL of fresh semen after liquefaction; to this, 950 μL of SDF was added, mixed, and left for 5–10 minutes prior to analysis. Ten microliters of this preparation was then placed on a clean slide, covered by a 22-mm × 22-mm coverslip, and examined at ×400 under phase–contrast optics. An HTM-SAM (Hamilton Thorne Research, Beverly, Mass) semiautomated counter was used to facilitate scoring and recording of data, with 100 cells on each slide being scored according to WHO guidelines.

Human Spermatozoa Preparation

In order to obtain an unselected population of cells, the remaining semen sample was placed into a centrifuge tube and spun at 500 × *g* for 5 minutes to pellet the spermatozoa. The seminal plasma was aspirated and kept aside for the measurement of total reactive antioxidant potential (TRAP assay). The pellet of spermatozoa was overlaid with 4 mL of Biggers-Whitten-Whitingham (BWW) medium (Biggers et al, 1971) containing 20 mM *N*-(2-hydroxyethyl) piperazine-*N′*-(2-ethan sulfonic acid) (HEPES; Gibco, United Kingdom) and 0.3% human serum albumin (HSA) and was processed by centrifuge again at 500 × *g* for 5 minutes. The supernatant was removed, and 2 mL BWW was added. A further centrifugation was performed, after which the cells were resuspended in 500 μL of BWW medium. Cell density was recorded with an improved Neubauer hemocytometer, and the correct amount of BWW was added to resuspend the cells at 20×10^6 mL. Aliquots were then taken for the following assays.

Total Reactive Antioxidant Potential

The TRAP assay was performed according to the method described by Smith (Smith et al, 1996) but with some modification. For the generation of peroxyl radicals, a solution of 30 mM 2,2-azobis-(2-amidinopropane) (ABAP) (Polysciences, Warrington, Pa) dissolved in phosphate-buffered saline (PBS) was used. Five minutes prior to each assay run, a 100-mM luminol stock solution in dimethyl sulfoxide was diluted 1 in 1000 in PBS/ABAP. Aliquots (400 μL each) of this mixture were placed in cuvettes in a Berthold 9505 biolumat luminometer and were allowed to equilibrate to the operating temperature (37°C) for 5 minutes while chemiluminescence was monitored. Seminal plasma was diluted 1:4 with PBS prior to addition to the ABAP/PBS solution. Once the peroxyl radical–generating system demonstrated a steady-state level of chemiluminescence, a 4-μL aliquot of the seminal plasma/PBS solution was added to quench the chemiluminescent signal. The time to 50% recovery of the initial signal was then calculated as the TRAP time. A standard curve was created for each run with 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carbonsaeure (TROLOX), and the results were expressed in TROLOX equivalents.

ROS Chemiluminescence

The methodology used was that described by Aitken et al (1992). Briefly, 200 μL of sperm suspension in BWW were placed in cuvettes of a luminometer with 2 μL of 100 μM luminol and 4 μL of a 2 mg/mL horseradish peroxidase solution (Sigma) to give a spontaneous chemiluminescent signal. After 10 minutes, a 1-μL aliquot of 10 mM *N*-formylmethionyl-leucyl-phenylalanine (FMLP) (Sigma) was added to detect leukocyte contamination, and after a further 10 minutes, 2 μL of a 1-mM stock solution of 12-myristate-13-acetate phorbol ester (PMA; Calbiochem, Nottingham, United Kingdom) was added to assess the total ROS-generating capacity of the ejaculate (leukocytes + spermatozoa). ROS chemiluminescence was then expressed as a 5-minute integral for each cycle (spontaneous, FMLP and PMA).

Computer-Assisted Sperm Assessment

The movement characteristics of the spermatozoa were examined with a Hamilton Thorne IVOS Motility Analyzer, version 10.5K, at an incubation temperature of 37°C. Each sample was loaded onto 20-μm-deep microcell slides (Microm, Thame, United Kingdom), and analysis was performed using a ×10 objective on five random fields from each sample. The analysis was performed in triplicate, and the mean of the three measurements was calculated. Parameter settings were as follows: frames acquired, 30; frame rate, 50 Hz; minimum contrast, 10; minimum cell size, 4; nonmotile head size, 12; nonmotile head intensity, 130; magnification, 1.96; static size limits, 0.59–2.87; and static intensity, 0.471–1.77. A wide range of different movement characteristics were assessed, including average path velocity (VAP); straight-line velocity (VSL), curvilinear velocity (VCL), linearity (LIN = VSL/VCL), straightness (STR = VSL/VAP), percentage rapid (VAP >25 μm/s), percentage medium speed (VAP = 10–24 μm/s), percentage slow (VAP = 1–9 μm/s), percentage static, percentage overall motility (rapid + medium speed + slow), percentage progressive motility (STR >75%), beat cross frequency (BCF), and amplitude of lateral sperm head displacement (ALH).

DNA Damage Assessment

Three assays for the determination of DNA damage were utilized: 1) single-cell gel electrophoresis (COMET) assay for single-stranded DNA; 2) ISNT utilizing streptavidin-fluorescein isothiocyanate (SA-FITC) for detection of biotin-16-dUTP incorporation; 3) modified ISNT assay with decondensation of spermatozoa prior to ISNT and utilization of an alkaline phosphatase (AP), 5-bromo-4-chloro-3-indoyl phosphate (BCIP):nitroblue tetrazolium (NBT) method for detection of incorporated biotin-16-dUTP (Twigg et al, 1997).

Single-Cell Gel Electrophoresis (Comet) Assay

The alkaline single-cell gel electrophoresis (comet) assay was based on existing methods described by Singh (1996), McKelvey-Martin et al (1993), and Hughes et al (1996), modified as indicated below. Unless otherwise stated, molecular-grade, DNAse-free reagents (Sigma, Poole, United Kingdom) were used throughout. Select microscope slides (Chance Proper, Smethwick, United Kingdom) were used for the assay, and each slide was prepared as follows: the slide was dipped in a solution of 1% multipurpose agarose (Boehringer Mannheim, Lewes, United Kingdom) dissolved in 0.01 M PBS and air-dried overnight at room temperature. One hundred sixty microliters of a

solution of 0.6% multipurpose agarose, dissolved in PBS, was then placed on the slide, covered with a coverslip measuring 24 × 50 mm (Chance Proper), and left to solidify at room temperature. Ten microliters of spermatozoa at 6.6 × 10^6 mL in BWW were then mixed with 75 μL of a 0.5% low-melt agarose solution (Amresco, Solon, Ohio) dissolved in PBS. This was then added to the slide, overlaid with a coverslip, and allowed to solidify on a prechilled (4°C) tray for several minutes. A final layer of 0.5% low-melt agarose was added to the slide and allowed to solidify at 4°C for at least 1 hour.

Following removal of the coverslip, the slides were immersed in lysis buffer (2.5 M NaCl, 100 mM ethylenediaminetetraacetic acid [EDTA], 10 mM Tris [hydroxymethyl] aminomethane hydrochloride [TRIS-HCl; Sigma], 10% dimethylsulfoxide [DMSO], and 1% Triton X-100, pH 10) for 1 hour at 4°C. The lysis solution was drained from the slides and replaced with a solution of proteinase K (Amresco) (100 μg/mL in 2.5 M NaCl, 100 mM EDTA, 10% DMSO, pH 7.4) and incubated overnight at 37°C. After draining the proteinase K solution, the slides were immersed in a horizontal gel tank filled with alkaline buffer (300 mM sodium hydroxide [NaOH], 1 mM EDTA, pH 12.3) for 20 minutes to allow the DNA to unwind. The buffer level was adjusted to a covering height of approximately 0.25 cm above the slides, and electrophoresis was carried out for 4 minutes at 25 V (0.862 V cm^{-1}). Slides were then placed in Coplin jars filled with 0.4 M Tris-HCl (pH 7.4) for 5 minutes and were washed three times with fresh buffer. After rinsing, the slides were drained and immersed in Coplin jars containing 100% ethanol for 5 minutes in order to precipitate the DNA and dehydrate the agarose. The slides were then air-dried overnight and stored in a box prior to scoring. Ethidium bromide was used to stain the sperm DNA (50 μL at 20 μg/mL dissolved in distilled water), which were then imaged on a Leitz fluorescent microscope (excitation filter 515–560 nm; dichroic filter, 580 nm; and suppression filter, 580 nm) connected to a charge couple device (CCD) camera. For each sample, four replicate slides were prepared, and 50 randomly selected cells were scored on each slide. The percentage of head DNA, tail DNA, and the olive tail moment (OTM) were evaluated with the Komet image analysis system, version 1.0 (Kinetic Imaging, Liverpool, United Kingdom) running on an IBM-compatible PC (Windows 3.1); the data were downloaded into an Excel spreadsheet (Microsoft, Wokingham, United Kingdom) for analysis.

In Situ Nick Translation of Spermatozoa

The method for ISNT was that described by Bianchi et al (Bianchi et al, 1993), with minor modifications. Spermatozoa were processed by centrifugation at 500 × *g* for 5 minutes, and the supernatant was removed. Fixative (ethanol:glacial acetic acid [Analar grade] 3:1) was added to bring the cells to a concentration of 10 × 10^6 mL. A 10-μL aliquot was then spotted onto a demarcated area on a clean microscope slide and allowed to air dry. Cells were rinsed once in tap water and twice in PBS before being overlaid with 50 μL DNA polymerase mix made up of 905 μL distilled water; 10 μL of 0.1 M dithiothreitol [DTT, Calbiochem]; 10 μL of 1 M MgSO4; 50 μL of 1 M Tris-HCl, pH 7.2; 10 μL of 1 mM biotin 16-dUTP (Boehringer Mannheim); 10 μL of a 1 mM dATP, dCTP, and dGTP mix (Promega, Southampton, United Kingdom); and 5 μL DNA polymerase 1 (Promega). Incubation was carried out at room temperature (25°C) for 30 minutes. After this, slides were rinsed again and overlaid with a solution of SA-FITC (900 μL distilled water; 90 μL 1 M Tris-HCl, pH 7.4; and 90 μL SAHR-FITC) for 30 minutes at room temperature. Once this incubation time had elapsed, the slides were rinsed as above and dried with a tissue. Ten microliters of mounting medium (3:1 distilled water:glycerol) were then placed on each slide and covered with a coverslip. Two hundred cells at ×400 magnification were scored for FITC fluorescence on a Leitz fluorescence microscope (excitation filter, 450–490 nm; dichroic filter, 510 nm; suppression filter, 520 nm), and the percentage was entered into an Excel (Microsoft) spreadsheet.

Modified In Situ Nick Translation of Spermatozoa

The protocol for this modified ISNT technique was that described by Twigg et al (1997). Briefly, spermatozoa were prepared at a concentration of 10 × 10^6 mL and were taken through two cycles of centrifugation and resuspension in BWW containing 6 mM ethylenediaminetetraacetic acid (EDTA; BDH, Poole, United Kingdom) and 2 mM dithiothreitol (DTT; Calbiochem), respectively. The cells were incubated at 37°C in 95% air and 5% CO_2 for 60 minutes, pelleted (500 × *g* for 5 minutes), and finally resuspended in 3:1 ethanol:glacial acetic acid (Analar grade; BDH). Ten microliters of this suspension were dropped onto a demarcated area of a clean glass microscope slide and allowed to air dry. A small volume of BWW containing 0.05% sodium dodecyl sulphate (SDS; Promega) was then added to each slide and allowed to stand for 2 minutes. In order to block any endogenous biotin/avidin-binding sites within the spermatozoa (Wood and Warnke, 1981), a blocking step was introduced that was composed of sequential 20-minute incubations with 0.001% biotin (Sigma) and 0.01% avidin (Sigma) in distilled water. ISNT was performed with the enzyme DNA polymerase 1 (Promega). Biotin incorporation was detected by an AP, BCIP:NBT (Boehringer Mannheim) method (De Jong et al, 1985). The slides were overlaid with a coverslip and incubated in a humidified chamber at room temperature for 16 hours. Following incubation, the slides were rinsed in tap water for 5 minutes and passed through 100% ethanol before being mounted under Pertex (Cellpath, Hemel Hempstead, United Kingdom). Fifty randomly selected sperm cells were captured at ×400 magnification on an Olympus BH2 microscope and were scored for optical density per sperm head by measuring each pixel of the digitized sperm head image on an arbitrary scale of 256 levels of gray on an IBM-compatible personal computer using Image Pro Plus for Windows, version 1.3.2. Data was saved in an Excel (Microsoft) spreadsheet before analysis.

Statistical Analysis

Data were analyzed with the Statistical Package for the Social Sciences software program (SPSS Inc., Chicago, Ill) on a Power Macintosh 8500 computer. The distributions of the variables were examined, and where necessary, they were normalized by log or square root transformation. Differences between groups were examined by means of the Mann-Whitney *U*-test or by unpaired *t*-test, as appropriate. Relationships between variables were examined by simple linear regression or by stepwise mul-

Table 1. *Semen quality assessed by WHO semen analysis and by CASA**

Variable	Donor Group Median	Donor Group IQR	Patient Group Median	Patient Group IQR	*P*
WHO semen profile					
Ejaculate volume (mL)	3	2–4.1	2.2	1.3–3.2	NS
Sperm concentration ($\times 10^6$ per mL)	94	76.8–123.8	40.5	26.4–81.5	0.0026
Overall motility (%) (= WHO criteria a+b+c)	63.17	58.1–67.0	42.6	31.7–54	0.0003
Progressive motility (%)	51.78	49.32–54.3	30.45	19.4–40.2	0.0008
Normal morphology (%)	43.3	38.2–51.3	40.4	32.3–54.9	NS
CASA profile					
Overall motility (%)	75.5	69.5–81.0	33	26.0–55.0	0.0011
Progressive motility (%)	32	26.0–38.5	10	6.0–20.0	0.001
ALH (μm)	3.85	3.6–4.6	4	3.1–4.7	NS
Linearity	46.5	45.0–48.5	44	37.0–48.0	NS
Straightness	72.5	70.0–73.0	69	63.0–71.0	NS
VAP (μm/s)	36.7	33.1–42.1	33.3	27.3–34.3	NS
VCL (μm/s)	56.35	51.6–64.5	53.4	45.6–60.3	NS
VSL (μm/s)	28.25	24.3–33.0	22.1	19.5–25.7	0.0183

* WHO indicates World Health Organization; CASA, computer-assisted sperm analysis; ALH, amplitude of lateral sperm head displacement; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity; NS, not significant; IQR, interquartile range.

tiple linear regression. Data are presented as medians with interquartile range (IQR).

Results

Semen Quality

Samples were collected from an unselected group of patients consulting for infertility and from a cohort of volunteer donors in order to provide a wide, dynamic range of semen quality attributes. In all, data on semen quality, ROS production, seminal plasma TRAP, and sperm DNA fragmentation were collected on 12 normal volunteer donors and on 29 male patients. In addition, CASA data were collected on a subgroup of 9 donors and 17 patients. All members of the donor group had normal values for sperm concentration (more than 20×10^6/mL), overall motility (>40% motile), and morphology (>30%; WHO, 1992), as defined for our local population (Irvine and Templeton, 1994). Three donor samples (25%) fell below the WHO threshold of 50% progressive motility. In the patient group, 7 of 29 (24%) were oligozoospermic (<20 $\times 10^6$/mL), 13 of 29 (44.8%) had low overall motility (<40%), 23 of 29 (79%) had low progressive motility by WHO standards (<50%), and 3 of 29 (10.3%) had teratozoospermia (<30% normal forms).

Descriptive statistics for both groups are presented in Table 1. The median sperm concentration for the donors was 94.0×10^6/mL (76.8–123.8, IQR), whereas that for the patient group was significantly lower at 40.5×10^6/mL (26.4–81.5 10^6/mL) (P = .0026). Similarly, the patient population were significantly more asthenozoospermic than the donor population, with a median progressive motility of 30.4% (19.4–40.2%), compared with 51.7% (49.3–54.3%) (P = .0003). There were no differences between the groups with respect to ejaculate volume or normal morphology. In the subgroup of subjects on whom CASA data was collected, the same differences were apparent, with the patient population having significantly lower values for sperm concentration and for overall and progressive motility compared with the donor cohort. There were no differences in any other attribute of sperm movement, with the exception of VSL, which was significantly lower in the patient cohort.

DNA Damage and Semen Quality

There were no significant differences between the groups in terms of ROS production, measured either in the resting state or after stimulation with FMLP or PMA, and there were no differences in the antioxidant potential of seminal plasma, measured either as TRAP time or as TROLOX equivalents (Table 2). In contrast, there were significant differences between the donor and patient groups with respect to DNA damage, with patients having higher levels of damage as determined by the comet assay. For the latter, the median percentage tail DNA was 10.44% (6.6–14.9) in the donor group and 19.8% (13.2–26.1) in the patient group (P = .002), and similar differences were seen for the OTM. Neither of the ISNT assays were significantly different between patient and donors.

Correlative analyses undertaken to examine the factors associated with DNA damage revealed a strong relationship with semen quality. A highly significant negative correlation was observed between sperm concentration (square root transformed) and DNA damage, as assessed by all three assays: comet ($R = -0.52$, P = .001), ISNT-decondensed ($R = -0.66$, $P < .0001$), and ISNT-con-

Table 2. *Reactive oxygen species production, seminal plasma antioxidant capacity, and DNA damage*

Variable	Donor Group		Patient Group		
	Median	IQR*	Median	IQR	*P*
Reactive oxygen production					
Spontaneous (cpm $\times 10^6$)	0.99	0.64–1.96	1.36	0.66–2.08	NS
FMLP stimulated (cpm $\times 10^6$)	0.65	0.39–1.07	0.65	0.37–1.60	NS
PMA stimulated (cpm $\times 10^6$)	2.31	0.70–9.67	1.65	0.61–2.93	NS
Total reactive antioxidant potential					
TRAP time	2.5	2.1–3.2	2.45	1.85–3.45	NS
TROLOX equivalent	2.85	2.45–3.15	2.4	1.85–3.65	NS
DNA damage: comet assay					
Median tail DNA (%)	10.44	6.6–14.9	19.81	13.2–26.1	0.002
Median olive tail moment	1.68	0.9–2.8	3.67	2.4–5.4	0.0035
DNA damage: in situ nick translation-condensed					
Cells positive (%)	2.03	0.99–3.11	3.63	1.78–7.22	NS
DNA damage: in situ nick translation-decondensed					
Median optical density	110.18	96.7–142.1	144.92	100.5–173.8	NS

* IQR indicates interquartile range; NS, not significant; FMLP, *N*-formylmethionyl-leucyl-phenylalanine; PMA, 12-myristate-13-acetate phorbol ester; TRAP, total reactive antioxidant potential; TROLOX, 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carbonsaeure.

densed ($R = -0.43$, $P = .006$; Figures 1A through C). When the number of morphologically normal, motile cells in the ejaculate was calculated (Glazener et al, 1987), it was found to be negatively correlated with DNA damage as measured by the single-cell gel electrophoresis and ISNT-decondensed assays ($R = -0.37$, $P = .026$; $R = -0.38$, $P = .016$); Figures 2A and B. However, although the same negative relationship was demonstrated with the ISNT-condensed assay, it was not statistically significant ($R = -0.20$, $P = .213$; Figure 2C).

Comparison of Techniques for Assessing DNA Damage

The relationships between DNA damage measured by the comet assay and ISNT-decondensed and ISNT-condensed are shown in Figure 2 (parts A, B, and C, respectively). It is clear that the comet assay had higher resolution for DNA fragmentation in that it allowed separation of subpopulations of individuals (Figure 2A). The donor population are clearly localized in the lower right-hand area of this scatterplot, with low levels of DNA damage and good semen quality. Several of the unselected patient population colocalized to this region, suggesting that these are normal men with both normal semen quality and low-level spermatozoal DNA fragmentation. A distinctly separate population of patients localized to the upper left region of the scatterplot, demonstrating poorer semen quality with higher levels of DNA fragmentation. Interestingly, a small population of individuals also displayed poor semen quality with low levels of DNA damage (Figure 2A).

While there were relationships between attributes of semen quality measured by CASA and DNA damage, these generally reflected the pattern that had emerged from the conventional semen profile of negative relationships between sperm number and motility. There were no meaningful relationships between observed levels of DNA damage and individual attributes of sperm movement (data not shown). Furthermore, no relationships were observed between levels of ROS production or seminal plasma antioxidant activity and DNA fragmentation in this population.

Prediction of DNA Damage From Conventional Semen Count

Finally, to examine the relationships between semen quality and DNA damage in more detail, a series of multiple linear regression analyses (with transformed variables where appropriate) were performed, the results of which are summarized in Tables 3 and 4. When DNA damage according to the comet assay was used as the dependent variable, with data from the WHO semen profile, the ROS chemiluminescence assay, and the TRAP assay entered as stepwise independent variables, it was seen that 25% of the variation in DNA damage could be accounted for by sperm concentration alone (Table 3). This relationship remained for the ISNT-decondensed assay, with 39% of the variability being explained by this variable alone. In a similar analysis for ISNT-condensed, 46% of the variation in DNA damage was accounted for with data derived from the conventional criteria of semen quality alone (morphology, sperm concentration, and progressive motility).

Similar analyses were then undertaken on the subset of patients and donors for whom CASA data was available. The results (Table 4) showed that a substantial proportion of the observed DNA damage could be accounted for by

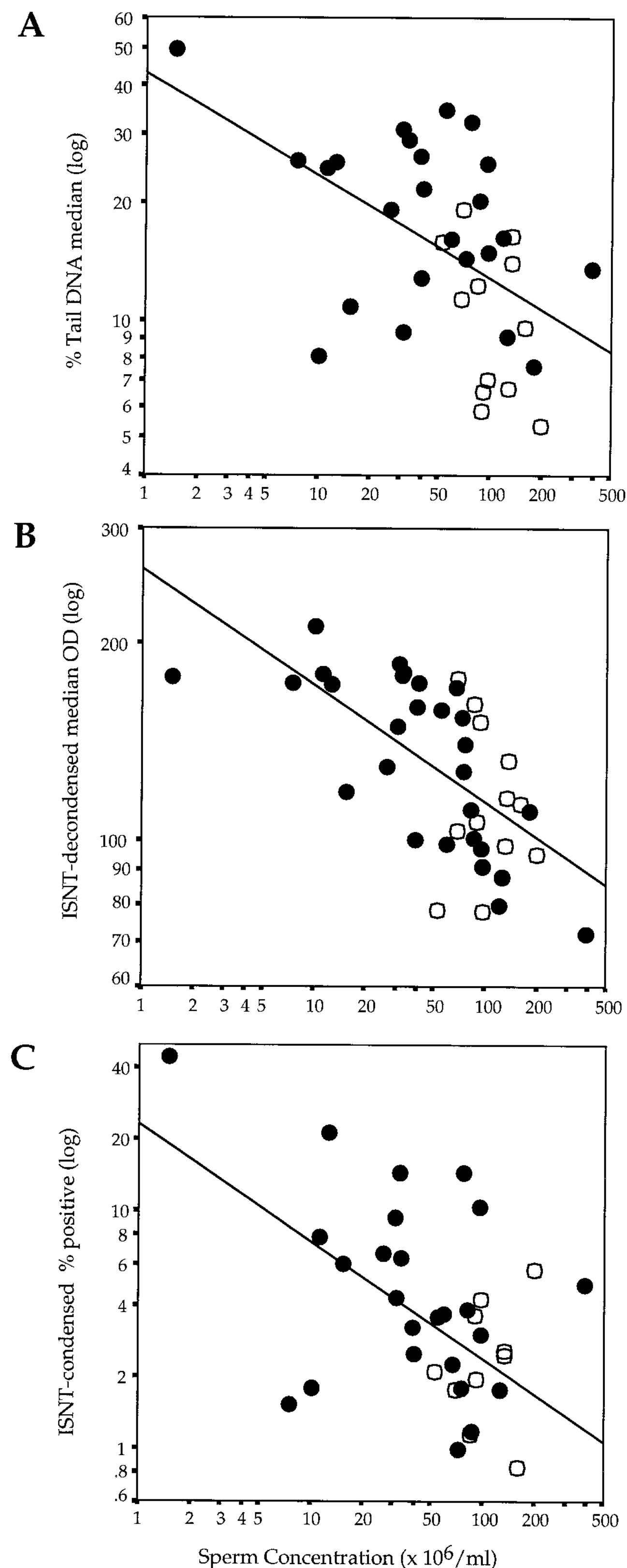


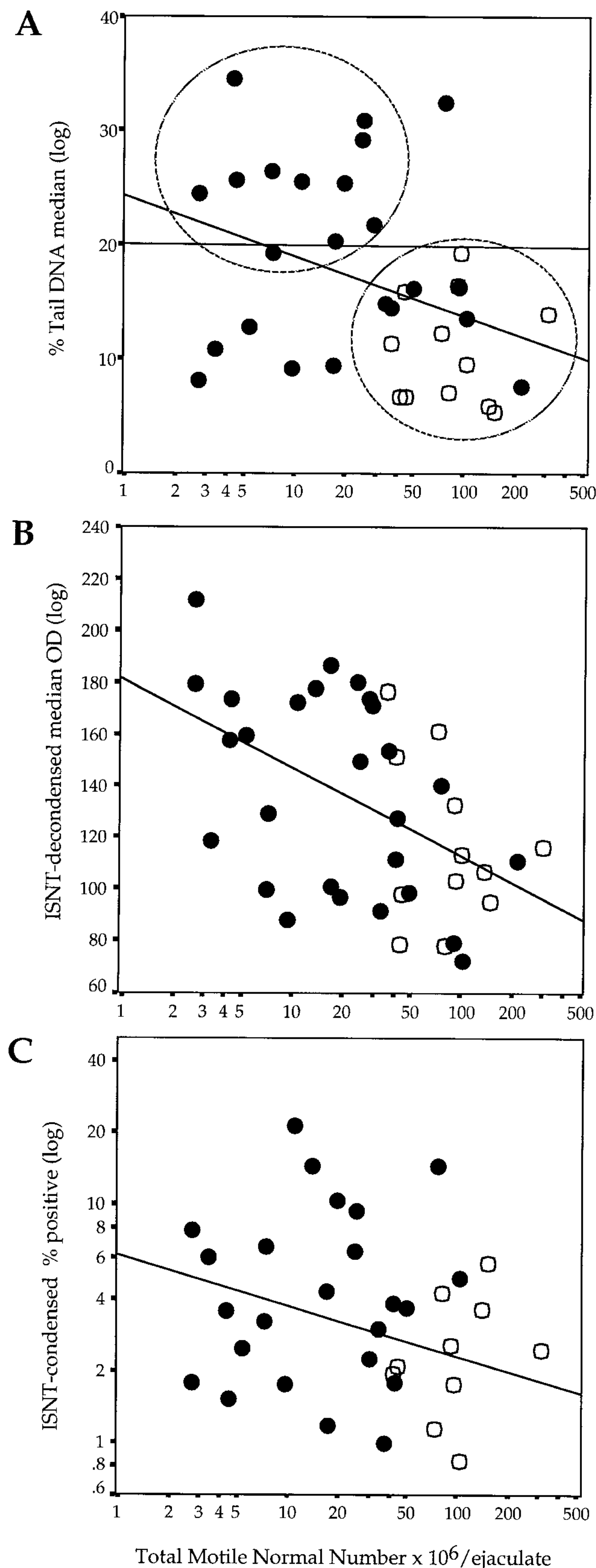
Figure 1. DNA damage for three assays plotted against sperm concentration. **(Panel A)** comet tail DNA percentage ($R = -0.52$, $P = .001$); **(Panel B)** in situ nick translation on decondensed spermatozoa (ISNT-decondensed) ($R = -0.66$, $P < .0001$); **(Panel C)** in situ nick translation on condensed spermatozoa (ISNT-condensed) ($R = -0.42$, $P = .006$). Open circles (○) represent normal donors; closed circles (●) represent patients.

the inclusion of additional variables. With the ISNT-decondensed assay, 37% of DNA damage was explained by sperm concentration alone, while in the case of the comet assay, 56% of DNA damage was explained by the combination of sperm concentration, spontaneous ROS chemiluminescence, and sperm movement (ALH). Similarly with the ISNT-condensed assay, almost 70% of the variability in DNA fragmentation could be explained by sperm concentration, morphology, sperm movement (VSL) and ROS-mediated chemiluminescence.

Discussion

The data presented here provide evidence of an important relationship between semen quality and DNA fragmentation. Using the comet assay as an extremely sensitive means of monitoring DNA strand breakage in human spermatozoa (Hughes et al, 1996) the impaired semen quality observed in the patient population (Table 1) was found to be associated with a significantly increased rate of DNA damage relative to a panel of normozoospermic donors ($P = .002$; Table 2). The attribute of semen quality that appeared to reflect the incidence of DNA damage most accurately was sperm concentration. On the basis of this criterion alone, 25% of the variance in DNA damage could be accounted for (Table 3). The correlations between semen quality and DNA damage were even more marked when the ISNT assays of DNA strand breakage were employed (Table 3). Thus, 39.1% of the variance in DNA damage observed with the ISNT-decondensed procedure could be accounted for by the sperm concentration in the ejaculate, while the corresponding figure for the ISNT-condensed assay was 46.4%, following incorporation of three semen quality variables into the regression equation describing sperm morphology, concentration, and progressive motility (Table 3).

Our finding of a negative relationship between DNA damage and semen quality is supported by data from other laboratories. For example, Sun and colleagues have recently demonstrated a significant negative correlation between semen quality (motility, morphology, and concentration in descending order of significance) and the presence of DNA strand breaks in spermatozoa from 285 men attending an assisted-conception clinic (Sun et al, 1997). However, this relationship was not found by Hughes et al (1996), who examined DNA fragmentation rates in the spermatozoa of normozoospermic fertile donors and asthenozoospermic infertile patients with a mod-



ified single-cell gel electrophoresis assay (Hughes et al, 1996), possibly because of differences in the composition of their study group. Nevertheless, these authors did find a difference between the donor and patient populations in terms of the sensitivity of the spermatozoa to DNA damage induced by exposure to hydrogen peroxide or X-ray irradiation; the DNA in the patients' spermatozoa were particularly sensitive to oxidative stress. The negative impact of oxidative stress in this context is reassuring because the damaging effect of hydrogen peroxide on DNA integrity has been confirmed in many other cell types (Martins and Meneghini, 1994; Zastawny et al, 1995) and by our own laboratory in work with spermatozoa utilizing the ISNT-decondensed methodology (Twigg et al, 1997).

The possible significance of oxidative DNA damage in the male germ line is supported by Kodama et al (1997), who found significantly higher levels of oxidative base damage in the spermatozoa of infertile patients compared with that of a control population. We have also recently shown that spermatozoa exposed to exogenous ROS or stimulated to produce high levels of endogenous ROS show higher rates of DNA strand breakage compared with untreated control samples (Twigg et al, 1997). In the present study, the significance of oxidative stress was suggested by the inclusion of spontaneous ROS generation in the multiple regression equations used to predict the DNA damage detected by the comet and ISNT-condensed assays (Table 4). Even so, the real importance of ROS as mediators of DNA damage in human spermatozoa might have been understated because of the methods used to prepare the spermatozoa. Thus, in order to generate material for a study of this kind, which required several different assays to be performed on the same sample, we elected to prepare an unselected population of cells by a washing and centrifugation protocol. This had the advantage of enhancing the number of spermatozoa available for analysis but had the disadvantage of generating an unselected population of cells, including a significant number of contaminating leukocytes. The presence of leukocytes would have obfuscated the detection of ROS production by the spermatozoa and would have obscured any relationships that might have existed between sperm-derived ROS and the induction of DNA damage (Twigg et al, 1998). In order to detect such relationships, a more selective sperm preparation technique would have been needed, such as Percoll density gradient centrifugation

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Figure 2. DNA fragmentation plotted against total motile normal sperm density per ejaculate. **(Panel A)** comet tail percentage DNA ($R = -0.37$, $P = .026$); **(Panel B)** in situ nick translation on decondensed spermatozoa (ISNT-decondensed) ($R = -0.38$, $P < .016$); **(Panel C)** in situ nick translation on condensed spermatozoa (ISNT-condensed). This relationship was not significant ($R = -0.20$, $P = .213$). Open circles (○) represent normal donors; closed circles (●) represent patients.

Table 3. *Attributes of semen qualtity associated with DNA damage in multiple linear regression*

Variables Offered	Variables Included	Regression Coefficient	Significance	Multiple R	R Square
Comet assay†					
WHO* semen profile	Sperm concentration‡	−0.498	0.0028	0.498	0.248
ROS assay					
TRAP assay					
In situ nick translation-decondensed					
WHO semen profile	Sperm concentration	−0.625	0.000	0.625	0.391
ROS assay					
TRAP assay					
In situ nick translation-condensed§					
WHO semen profile	Normal morphology	0.418	0.0035	0.681	0.464
ROS assay	Sperm concentration	−0.296	0.055		
TRAP assay	Progressive motility	−0.374	0.018		

* WHO indicates World Health Organization; ROS, reactive oxygen species; TRAP, total reactive antioxidant potential.
† Percentage tail DNA = log 10 transformed.
‡ Sperm concentration = square root transformed.
§ In situ nick translation = log 10 transformed.

followed by treatment with anti-CD45–coated magnetic beads (Aitken et al, 1989b; 1996; Agarwal et al, 1994). Further studies are clearly required to fully establish the cause of the excessive DNA damage detected in the spermatozoa of infertile men. However, because both ROS generation and oxidative DNA base damage are elevated in the spermatozoa of infertile patients (Aitken et al, 1992; Kodama et al, 1997) and because ROS production was selected as a predictor of DNA damage in the present study, a rational hypothesis would be that the etiology of this damage involves oxidative stress within the male reproductive tract. One possibility would be the release, through defective spermiation of immature spermatozoa carrying excessive residual cytoplasm (Gomez et al, 1996), which have been shown to suffer from excessive exogenous oxidative stress (Huszar and Vigue, 1994; Huszar et al, 1998).

Given the existence of high levels of DNA fragmentation in the spermatozoa of infertile patients, important questions remain with regard to the optimal means of detecting this damage and its clinical significance. In terms of methodology, three different techniques were employed in the present study to detect DNA strand breakage in human spermatozoa. The comet assay has been shown to be one of the most sensitive assays for the detection of DNA damage (Leroy et al, 1996; Singh, 1996). Sensitivity to the detection of single-strand DNA breakage comes from the use of alkaline lysis buffer, which reverses DNA supercoiling and separates the DNA duplex into single strands. Further sensitivity to subtle changes in DNA is gained by incubation of the cells in the presence of proteinase K, which removes protamines that otherwise impede DNA migration through the agarose. Minor variations in the protocol employed during this assay, such as the inclusion of antioxidants in electrophoresis buffer, alterations in electrophoresis time, and differences in pH can all influence the sensitivity of the assay. The comet assay may even overestimate true DNA strand breakage in spermatozoa because of artificial damage induced at alkali-labile sites within the DNA strand (Singh et al, 1989). Nevertheless, in the context of human spermatozoa, this assay would appear to be the most sensitive of all the procedures assessed for detecting DNA fragmentation, since this technique alone was able to discriminate the central trends in DNA damage between the patient and donor populations (Table 2).

Although the comet assay was extremely sensitive, it did not show the same levels of correlation with semen quality that were recorded for the ISNT-condensed procedure (Table 4). Almost 70% of the variation in DNA damage recorded with the ISNT-condensed assay could be accounted for on the basis of semen quality as reflected in sperm concentration, sperm morphology, VSL, and spontaneous ROS generation. The high correlations observed between the ISNT-condensed assay and semen quality may reflect the fact that this assay measures more than just DNA damage; it also provides an assessment of the efficiency of DNA compaction. It has been suggested by other groups that clinically significant differences exist between cells in sperm chromatin packaging (Sakkas et al, 1995, 1996), an observation that would be consistent with the finding that sperm from infertile men are more susceptible to DNA damage induced by exogenous factors such as irradiation or hydrogen peroxide (Hughes et al, 1996). The ISNT-condensed assay is reliant on the access DNA polymerase 1 gains to the genome as a result of incomplete compaction of the sperm nucleus, the level of DNA nick-end labeling being highly correlated with

Table 4. *Attributes of sperm qualtity associated with DNA damage in multiple linear regression for individuals with CASA* analysis*

Variables Offered	Variables Included	Regression Coefficient	Significance	Multiple R	R Square
Comet Assay†					
WHO semen profile	ALH	0.686	0.0023	0.748	0.56
CASA	Sperm concentration‡	−0.665	0.0041		
ROS assay	Spontaneous ROS§	−0.381	0.0527		
TRAP assay					
In situ nick translation-decondensed					
WHO semen profile	Sperm concentration	−0.608	0.0027	0.608	0.369
CASA					
ROS assay					
TRAP assay					
In situ nick translation-condensed‖					
WHO semen profile	Sperm concentration	−0.452	0.0088	0.833	0.693
CASA	Normal morphology	0.430	0.0131		
ROS assay	VSL	−0.560	0.0123		
TRAP assay	Spontaneous ROS	−0.375	0.0367		

* CASA indicates computer-assisted sperm analysis; WHO, World Health Organization; ROS, reactive oxygen species; TRAP, total reactive antioxidant potential; ALH, amplitude of lateral head displacement; VSL, straight-line velocity.
† Percentage tail DNA = log 10 transformed.
‡ Sperm concentration = square root transformed.
§ Spontaneous ROS production = log 10 transformed.
‖ In situ nick translation = log 10 transformed.

the levels of sperm nucleus protamination (Manicardi et al, 1995). Therefore, this assay describes a relationship between semen quality and DNA damage because it incorporates a measure of chromatin condensation that in turn reflects the quality of the processes controlling the differentiation and maturation of the spermatozoa. However, the major problem with this procedure is that it has a very narrow dynamic range, given that less than 10% of all spermatozoa are labeled, even in the patient group (Table 2).

In order to increase the sensitivity of the ISNT assay, we have introduced a variation of this technique involving the chemical decondensation of the chromatin prior to the labeling procedure (Twigg et al, 1997). This modification means that errors in DNA compaction are no longer being detected by the assay and that as a result, the correlations with semen quality are less apparent (Table 4). However, in this form, the ISNT assay is more sensitive to DNA damage per se and can detect strand breakage induced in the spermatozoa after compaction (by, for example, exposure to hydrogen peroxide) that would have been missed by the conventional ISNT assay (Twigg et al, 1997; Twigg, unpublished data).

The finding that poor semen quality is associated with DNA fragmentation in the spermatozoa may have implications for individuals being treated with assisted-conception technology, particularly ICSI. We have recently demonstrated that a loss of DNA integrity has no impact on the ability of the spermatozoon to fertilize the oocyte after ICSI (Twigg et al, 1997). It has been suggested that embryos conceived by ICSI may be more likely to suffer an increased rate of preclinical and clinical pregnancy loss than those conceived by standard IVF (Bar-Hava et al, 1997), although until recently, data on the rates of birth abnormalities was reassuring, except for the increased risk for sex chromosome abnormality (Bonduelle et al, 1996a,b). This debate has now been reopened by a reinterpretive paper published by Kurinczuk and Bower (1997). Clarification of whether there is an increased risk of abnormality for offspring conceived by ICSI will only be refuted or confirmed over time, but in addition to possible abnormalities apparent at birth or soon thereafter, there may be risks in later childhood arising from genetic damage to spermatozoa. It is known, for example, that smokers carry higher levels of oxidative damage in their spermatozoa (Fraga et al, 1996; Shen et al, 1997; Sun et al, 1997), which is associated with a highly significant increased risk of childhood cancer in their offspring (Ji et al, 1997; Sorahan et al, 1997). Hence, it is reasonable to speculate that the use of DNA-damaged spermatozoa to effect fertilization during ICSI may additionally predispose the offspring to a risk of serious disease in later life.

Further research is therefore warranted to establish the long-term safety of microassisted fertilization, and consideration should be given to incorporating these concerns into the counseling of couples going forward for this treatment. Establishing the etiology of this damage may serve as a basis upon which to provide medical treatment

(Kodama et al, 1997) to individuals with high baseline damage prior to assisted conception.

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References

Agarwal A, Ikemoto I, Loughlin KR. Effect of sperm washing on levels of reactive oxygen species in semen. *Arch Androl* 1994;33:157–162.

Aitken RJ, Buckingham DW, Brindle J, Gomez E, Baker HWG, Irvine DS. Analysis of sperm movement in relation to the oxidative stress created by leukocytes in washed sperm preparations and seminal plasma. *Hum Reprod* 1995;10:2061–2071.

Aitken RJ, Buckingham DW, West KM. Reactive oxygen species and human spermatozoa—analysis of the cellular mechanisms involved in luminol-dependent and lucigenin-dependent chemiluminescence. *J Cell Physiol* 1992;151:466–477.

Aitken RJ, Buckingham DW, West K, Brindle J. On the use of paramagnetic beads and ferrofluids to assess and eliminate the leukocytic contribution to oxygen radical generation by human sperm suspensions. *Am J Reprod Immunol* 1996;35:541–551.

Aitken RJ, Clarkson JS, Fishel S. Generation of reactive oxygen species, lipid-peroxidation, and human-sperm function. *Biol Reprod* 1989a;41: 183–197.

Aitken RJ, Clarkson JS, Hargreave TB, Irvine DS, Wu FCW. Analysis of the relationship between defective sperm function and the generation of reactive oxygen species in cases of oligozoospermia. *J Androl* 1989b;10:214–220.

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

Aitken RJ, West K, Buckingham D. Leukocytic infiltration into the human ejaculate and its association with semen quality, oxidative stress, and sperm function. *J Androl* 1994;15:343–352.

Altman SA, Zastawny TH, RandersEichhorn L, Cacciuttolo MA, Akman SA, Dizdaroglu M, Rao G. Formation of DNA-protein cross-links in cultured mammalian cells upon treatment with iron ions. *Free Rad Biol Med* 1995;19:897–902.

Baccetti B, Collodel G, Piomboni P. Apoptosis in human ejaculated sperm cells. *J Submicrosc Cytol Pathol* 1996;28:587–596.

Bar-Hava I, Ashkenazi J, Shelef M, Schwartz A, Brengauz M, Feldberg D, Orvieto R, Ben-Rafael Z. Morphology and clinical outcomes of embryos after in vitro fertilization are superior to those after intracytoplasmic sperm injection. *Fertil Steril* 1997;68:653–657.

Bianchi PG, Manicardi GC, Bizzaro D, Bianchi U, Sakkas D. Effect of deoxyribonucleic acid protamination on fluorochrome staining and in situ nick-translation of murine and human mature spermatozoa. *Biol Reprod* 1993;49:1083–1088.

Biggers J, Whitten W, Whittingham D. *The Culture of Mouse Embryos in Vitro*. San Francisco: Freeman; 1971.

Bonduelle M, Legein J, Buysse A, Van Assche E, Wisanto A, Devroey P, Van Steirteghem AC, Liebaers I. Prospective follow-up study of 423 children born after intracytoplasmic sperm injection. *Hum Reprod* 1996a;11:1558–1564.

Bonduelle M, Wilikens A, Buysse A, Van Assche E, Wisanto A, Devroey P, Van Steirteghem AC, Liebaers I. Prospective follow-up study of 877 children born after intracytoplasmic sperm injection (ICSI), with ejaculated epididymal and testicular spermatozoa and after replacement of cryopreserved embryos obtained after ICSI. *Hum Reprod (suppl 4)* 1996b;11:131–155.

Cummins JM, Jequier AM. Concerns and recommendations for intracytoplasmic sperm injection (ICSI) treatment. *Hum Reprod* 1995;10: 138–143.

De Jong ASH, Van Kessel-Van Vark M, Raap AK. Sensitivity of various visualisation methods for peroxidase and alkaline phosphatase activity in immunoenzyme histochemistry. *Histochem J* 1985;17:1119–1130.

Dizdaroglu M. Oxidative damage to DNA in mammalian chromatin. *Mutat Res* 1992;275:331–342.

Foresta C, Rossato M, Garolla A, Ferlin A. Male infertility and ICSI: are there limits? *Hum Reprod* 1996;11:2347–2348.

Fraga CG, Motchnik PA, Wyrobek AJ, Rempel DM, Ames BN. Smoking and low antioxidant levels increase oxidative damage to sperm DNA. *Mutat Res* 1996;351:199–203.

Glazener CM, Kelly NJ, Weir MJ, David JS, Cornes JS, Hull MG. The diagnosis of male infertility—prospective time-specific study of conception rates related to seminal analysis and post-coital sperm-mucus penetration and survival in otherwise unexplained infertility. *Hum Reprod* 1987;2:665–671.

Gomez E, Buckingham DW, Brindle J, Lanzafame F, Irvine DS, Aitken RJ. Development Of an image-analysis system to monitor the retention of residual cytoplasm by human spermatozoa—correlation with biochemical markers of the cytoplasmic space, oxidative stress, and sperm function. *J Androl* 1996;17:276–287.

Hughes CM, Lewis SEM, McKelvey-Martin VJ, Thompson W. A comparison of baseline and induced DNA damage in human spermatozoa from fertile and infertile men, using a modified comet assay. *Mol Reprod Dev 1996*;2:613–619.

Hull MGR. The causes of infertility and relative effectiveness of treatment. In: Templeton AA, Drife JO, eds. *Infertility*. Berlin: Springer-Verlag; 1992:33–57.

Huszar G, Patrizio P, Vigue L, Willets M, Wilker C, Adhoot D, Johnson L. Cytoplasmic extrusion and the switch from creatine kinase B to M isoform are completed by the commencement of epididymal transport in human and stallion spermatozoa. *J Androl* 1998;19:11–20.

Huszar G, Vigue L. Correlation between the rate of lipid peroxidation and cellular maturity as measured by creatine kinase activity in human spermatozoa. *J Androl* 1994;15:71–77.

Irvine DS, Templeton AA. Donor insemination. In: Hargreave, TB, ed. *Male Infertility*. Edinburgh, United Kingdom: Churchill Livingstone; 1994:427–451.

Iwasaki A, Gagnon C. Formation of reactive oxygen species in spermatozoa of infertile patients. *Fertil Steril* 1992;57:409–416.

Ji BT, Shu XO, Linet MS, Zheng W, Wacholder S, Gao YT, Ying DM, Jin F. Paternal cigarette smoking and the risk of childhood cancer among offspring of nonsmoking mothers. *J Natl Cancer Inst* 1997; 89:238–244.

Kodama H, Yamaguchi R, Fukuda J, Kasai H, Tanaka T. Increased oxidative deoxyribonucleic acid damage in the spermatozoa of infertile male patients. *Fertil Steril* 1997;68:519–524.

Kurinczuk J, Bower C. Birth defects in infants conceived by intracytoplasmic sperm injection: an alternative interpretation. *Br Med J* 1997; 315:1260–1263.

Lalwani S, Sayme N, Vigue L, Corrales M, Huszar G. Biochemical markers of early and late spermatogenesis: relationship between the lactate dehydrogenase-X and creatine kinase-M isoform concentrations in human spermatozoa. *Mol Reprod Dev* 1996;43:495–502.

Leroy T, Van Hummelen P, Anard D, Castelain P, KirschVolders M, Lauwerys R, Lison D. Evaluation of three methods for the detection of DNA single-strand breaks in human lymphocytes: alkaline elution, nick translation, and single-cell gel electrophoresis. *J Toxicol Env Health* 1996;47:409–422.

Lloyd DR, Phillips DH, Carmichael PL. Generation of putative intras-

trand cross-links and strand breaks in DNA by transition metal ion-mediated oxygen radical attack. *Chem Res Toxicol* 1997;10:393–400.

Manicardi G, Bianchi P, Pantano S, Azzoni P, Bizzaro D, Bianchi U, Sakkas D. Presence of endogenous nicks in DNA of ejaculated human spermatozoa and its relationship to chromonycin A3 accessibility. *Biol Reprod* 1995;52:864–867.

Martins EAL, Meneghini R. Cellular DNA damage by hydrogen peroxide is attenuated by hypotonicity. *Biochem J* 1994;299:137–140.

McKelvey-Martin VJ, Green MHL, Schmezer P, Pool-Zobel BL, De Meo MP, Collins A. The single cell gel electrophoresis assay (comet assay): a European review. *Mutat Res* 1993;288:47–63.

Palomba L, Brambilla L, Brandi G, Sestili P, Cattabeni F, Cantoni O. Low levels of hydrogen peroxide and L-histidine induce DNA double-strand breakage and apoptosis. *Eur J Pharmacol* 1996;318:167–173.

Sakkas D, Manicardi G, Bianci P, Bizzaro D, Bianchi U. Relationship between the presence of endogenous nicks and sperm chromatin packaging in maturing and fertilising mouse spermatozoa. *Biol Reprod* 1995;52:1149–1155.

Sakkas D, Urner F, Bianchi PG, Bizzaro D, Wagner I, Jaquenoud N, Manicardi G, Campana A. Sperm chromatin anomalies can influence decondensation after intracytoplasmic sperm injection. *Hum Reprod* 1996;11:837–843.

Schmidt L, Münster K, Helm P. Infertility and the seeking of infertility treatment in a representative population. *Brit J Obstet Gynaecol* 1995; 102:978–984.

Shen HM, Chia SE, Ni ZY, New AL, Lee BL, Ong CN. Detection of oxidative DNA damage in human sperm and the association with cigarette smoking. *Reprod Toxicol* 1997;11:675–680.

Singh NP. Microgel electrophoresis of DNA. In: G. P. Pfeifer, ed. *Technologies for Detection of DNA Damage and Mutations*. New York: Plenum Press; 1996:3–24.

Singh NP, Danner DB, Tice RR, McCoy MC, Collins GD, Schneider EL. Abundant alkali labile sites in DNA of human and mouse sperm. *Exp Cell Res* 1989;184:461–470.

Smith R, Vantman D, Ponce J, Escobar J, Lissi E. Total antioxidant capacity of human seminal plasma. *Hum Reprod* 1996;11:1655–1660.

Sorahan T, Lancashire RJ, Hulten MA, Peck I, Stewart AM. Childhood cancer and parental use of tobacco: deaths from 1953 to 1955. *Br J Cancer* 1997;75:134–138.

Sukcharoen N, Keith J, Irvine DS, Aitken RJ. Prediction of the in-vitro fertilization (IVF) potential of human spermatozoa using sperm function-tests—the effect of the delay between testing and IVF. *Hum Reprod* 1996;11:1030–1034.

Sun JG, Jurisicova A, Casper RF. Detection of deoxyribonucleic acid fragmentation in human sperm: correlation with fertilization in vitro. *Biol Reprod* 1997;56:602–607.

Templeton AA. The epidemiology of infertility. In: Templeton AA, Drife JO, eds. *Infertility*. Berlin: Springer-Verlag; 1992:23–34.

Thonneau P, Marchand S, Tallec A, Ferial M-L, Ducot B, Lansac J, Lopes P, Tabaste J-M, Spira A. Incidence and main causes of infertility in a resident population (1,850,000) of three French regions (1988–1989). *Hum Reprod* 1991;6:811–816.

Twigg J, Irvine D, Aitken R. Oxidative damage to DNA in human spermatozoa does not preclude pronucleus formation at ICSI. *Hum Reprod* 1997;13:1864–1871.

Twigg J, Irvine DS, Houston P, Fulton N, Michael L, Aitken RJ. Iatrogenic DNA damage induced in human spermatozoa during sperm preparation: protective significance of seminal plasma. *Mol Hum Reprod* 1998;4:439–445.

Van Steirteghem A, Liu J, Joris H, Nagy Z, Janssenswillen C, Tournaye H, Derde M, Van Assche E, Devroey P. Higher success rate by intracytoplasmic sperm injection than by subzonal insemination. Report of a second series of 300 consecutive treatment cycles. *Hum Reprod* 1993a;8:1055–1060.

Van Steirteghem A, Nagy Z, Joris H, Liu J, Staessen C, Smitz J, Wisanto A, Devroey P. High fertilization and implantation rates after intracytoplasmic sperm injection. *Hum Reprod* 1993b;8:1061–1066.

Wisanto A, Bonduelle M, Camus M, Tournaye H, Magnus M, Liebaers I, Van Steirteghem A, Devroey P. Obstetric outcome of 904 pregnancies after intracytoplasmic sperm injection. *Hum Reprod* 1996;11: 121–129.

Wood G, Warnke R. Suppresion of endogenous avidin-binding activity in tissues and its relevance to biotin-avidin detection systems. *J Histochem Cytochem* 1981;29:1196–1204.

World Health Organization (1992). *WHO Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction*. 3rd ed. Cambridge, United Kingdom: Cambridge University Press; 1987.

Zastawny TH, Altman SA, Randers-Eichhorn L, Madurawe R, Lumpkin JA, Didzaroglu M, Rao G. DNA base modifications and membrane damage in cultured mammalian cells treated with iron ions. *Free Rad Biol Med* 1995;18:1013–1022.