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An algorithm to predict pregnancy in assisted reproduction

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BACKGROUND: Male fertility potential cannot be measured by conventional parameters for the assisted reproduction technique; ICSI. This study determines the relationship between testicular and ejaculated sperm mitochondrial (mt) DNA deletions, nuclear (n) DNA fragmentation, and fertilization and pregnancy rates in ICSI. **METHODS:** Ejaculated sperm were obtained from 77 men and testicular sperm from 28 men with obstructive azoospermia undergoing ICSI. Testicular sperm were retrieved using a Trucut needle. mtDNA was analysed using a long PCR. The alkaline Comet assay determined nDNA fragmentation. **RESULTS:** Of subjects who achieved a pregnancy (50%) using testicular sperm, only 26% had partners' sperm with wild-type (WT) mtDNA. Of pregnant subjects (38%) using ejaculated sperm, only 8% had partner sperm with WT mtDNA. In each, the successful group had less mtDNA deletions and less nDNA fragmentation. There were inverse relationships between pregnancy and mtDNA deletion numbers, size and nDNA fragmentation for both testicular and ejaculated sperm. No relationships were observed with fertilization rates. An algorithm for the prediction of pregnancy is presented based on the quality of sperm nDNA and mtDNA. **CONCLUSION:** In both testicular and ejaculated sperm, mtDNA deletions and nDNA fragmentation are closely associated with pregnancy in ICSI.

Key words: fertilization/human testicular and ejaculated sperm/mitochondrial DNA/nuclear DNA/pregnancy

Introduction

Sperm nuclear (n) DNA integrity is increasingly recognized as a useful indicator of male fertility potential. Studies have shown that sperm with higher levels of nDNA damage have lower fertilization rates after IVF (Sun *et al.*, 1997) and ICSI (Lopes *et al.*, 1998; Esterhuizen *et al.*, 2000). Sperm with nDNA damage above a certain threshold are associated with a longer time to conceive in otherwise apparently fertile couples (Evenson *et al.*, 1999; Spano *et al.*, 2000), and a higher miscarriage rate (Evenson *et al.*, 1999). Infertile men have also been shown to have more nDNA anomalies than sperm donors and fertile men (Sakkas *et al.*, 1996; Irvine *et al.*, 2000; Zini *et al.*, 2001). These defects include poor DNA packaging (Sakkas *et al.*, 1996; Filatov *et al.*, 1999), increased strand breaks (Lopes *et al.*, 1998; Irvine *et al.*, 2000), greater susceptibility to DNA denaturation (Evenson *et al.*, 1999) and a higher incidence of chromosomal abnormalities (Moosani *et al.*, 1995). In ICSI cycles, DNA damage has been shown to be associated with impaired embryo cleavage (Morris *et al.*, 2002). In addition, higher rates of pregnancy loss have been documented in ICSI than in IVF (Bar-Hava *et al.*, 1997). As these pregnancies were almost inevitably achieved with sperm of poor quality that would have been incapable of fertilizing an oocyte naturally, there may well be a link with sperm nDNA damage. Yet, because of the success of ICSI in bypassing

rather than addressing the problem of poor sperm quality, such defective sperm are now routinely used in clinical treatments. Since ICSI does not require strongly motile sperm, its use has been expanded to incorporate immature sperm from the testes and epididymis. As yet, there have been few studies assessing the DNA integrity of sperm from these sources. Thus, potentially, sperm with damaged DNA are currently being used indiscriminately in assisted reproduction.

A useful assay for the detection of DNA damage in ejaculated and testicular sperm is the single-cell gel electrophoresis assay, also known as the alkaline 'Comet assay' (Donnelly *et al.*, 1999). The Comet assay has been used *in vitro* and *in vivo* to assess DNA damage and repair induced by various agents in a wide variety of mammalian cells (Singh *et al.*, 1988; Tice *et al.*, 1990; Olive *et al.*, 1998). It has become so useful that its applications range from studies of effects of UV radiation, carcinogens, radiotherapy and toxicology to tumour suppression (Fairbairn *et al.*, 1995). This is the first technique to allow measurement of nDNA damage in single cells. It does so rapidly (McKelvey-Martin *et al.*, 1993; Fairbairn *et al.*, 1995), reproducibly (Hughes *et al.*, 1997) and with higher sensitivity than alkaline elution or nick translation (Leroy *et al.*, 1996; Irvine *et al.*, 2000). One of its unique and powerful features is its ability to characterize the range of responses to DNA damage within a heterogeneous population

of cells such as that of sperm by measuring damage within individual cells. The assay is simple, inexpensive and requires few cells for analysis.

The nucleus is not the sperm's only source of DNA; the mitochondrion also has its own genome. During recent years, mitochondrial (mt) DNA mutations have been identified in numerous diseases (Wallace *et al.*, 1994). mtDNA changes have also been identified as the origin of ageing (Shigenaga *et al.*, 1994), and more recently as a cause of male infertility (Cummins *et al.*, 1994; St John *et al.*, 1997). mtDNA is also an indicator of the health of the sperm. mtDNA deletions lead to deficient oxidative phosphorylation that, in turn, causes abnormal metabolism and inadequate sperm motility. Despite its importance, mtDNA has not yet been fully characterized in human sperm, nor has any potential relationship with successful assisted reproduction treatment (ART) outcome been determined.

In this study we used a long PCR (LPCR) (Lestienne *et al.*, 1997) to determine the number and size of mtDNA deletions in testicular and ejaculated sperm and a modified alkaline Comet assay (Hughes *et al.*, 1996; Donnelly *et al.*, 1999) to assess nDNA. We then investigated the relationship between mtDNA, nDNA, and fertilization and pregnancy rates following ICSI with ejaculated and testicular (TICSI) sperm to assess the value of mtDNA and nDNA damage separately and in combination as prognostic tests for treatment outcomes.

Materials and methods

Subjects

Testicular biopsies were obtained from men with obstructive azoospermia ($n = 28$) attending the Regional Fertility Centre, Belfast. Patients with obstructive azoospermia were identified on the basis of two azoospermic semen analyses, normal testicular histology and volume, normal FSH levels and a normal karyotype.

Semen samples were obtained from male partners of couples attending the Regional Fertility Centre, Royal Maternity Hospital, Belfast for ICSI ($n = 77$). All men were the partners of women who had failed to conceive after at least 2 years of unprotected intercourse. Twenty men had asthenozoospermia, three had failed fertilization in a previous cycle of IVF and the remainder had oligoasthenoteratozoospermia.

Samples were obtained on the day of oocyte retrieval following a recommended 48–96 h of sexual abstinence and included in this study if there was a sufficient quantity of sperm left over after therapeutic insemination. Couples who were attending for ICSI using donor sperm or oocyte donation were not included. Informed written consent for participation was obtained. The project was approved by the Queen's University Belfast Research and Ethics Committee and was in accordance with The Declaration of Helsinki as revised in 1983.

Testicular sperm retrieval and preparation (Steele *et al.*, 2000)

Briefly, the spermatic cord was located and 10 ml of 0.5% bupivacaine injected around it. The epididymis was then located and a 21 G butterfly needle used to aspirate the sperm into Biggers–Whitten–Whittingham (BWW) (Biggers *et al.*, 1971) media. This was spun at 110 g for 10 min to pellet the sperm. The supernatant was discarded and the pellet resuspended in BWW for analysis. A testicular biopsy was performed by passing a 14 G Trucut biopsy needle (Baxter Healthcare Ltd, Thetford, UK) into the testis after the skin over the site

had been anaesthetized with 2 ml of 1% lignocaine. The biopsy was transferred into BWW medium.

Testicular sperm were retrieved from the seminiferous tubules by 'milking' the tubular contents with size 5 jeweller's forceps into BWW, under a dissecting microscope. The contents of the seminiferous tubules were then centrifuged at 1000 g for 10 min to remove debris and the sperm pellet resuspended in BWW (300 µl).

ICSI procedure

All ICSI cycles were performed according to routine procedures. Ovulation induction was achieved with Metrodin HP (Serono, Welwyn Garden City, UK) following a long protocol of pituitary desensitization with buserelin acetate (Suprefact; Hoechst, Hounslow, UK or Synarel, Syntex Pharmaceuticals Ltd, Maidenhead, UK). HCG (Pregnyl, Organon, UK) was administered when there were at least four follicles of diameter >17 mm, 36 h before oocyte retrieval. Mature, metaphase II oocytes obtained by vaginal ultrasound-guided aspiration were cultured in universal IVF medium, Medicult 10310500 (Biotipp; Cahir, Co., Tipperary, Ireland) at 37°C, 5% CO₂ in air. The injection procedure has been described in detail previously (Van Steirteghem *et al.*, 1993). In brief, the injection pipette was filled with a small amount of polyvinylpyrrolidone (PVP) (Sigma, Poole, UK) and a free, motile sperm was aspirated into the pipette, head-first from 5 µl of universal IVF medium. The single sperm was then placed, without any cell debris, in another droplet of PVP and immobilized. The sperm was aspirated again into the injection pipette tail-first and injected into an oocyte. Fertilization was recorded 12–16 h after injection if two pronuclei were detected. In each case, two embryos were transferred into the uterine cavity after an additional 24 h. Luteal support was provided by Progynova (8 mg) and Cyclogest by vaginal pessary twice daily (400 mg). An intrauterine pregnancy with fetal heart beat was confirmed by ultrasound at 6 weeks.

Preparation of ejaculated semen using Puresperm

In the ejaculated sperm group, each patient produced a split ejaculate in order to collect the majority of the sperm in the first container without dilution by seminal fluid. The sperm were prepared for oocyte injection using a discontinuous (95.0–47.5%) PureSperm™ (Hunter Scientific Ltd, Saffron Walden, UK) gradient. Semen (1 ml) was layered on top of the gradient and centrifuged at 500 g for 20 min, followed by resuspension in fresh media (2 ml) and a further concentration step by centrifugation at 250 g for 10 min. The final sperm preparation was suspended in a suitable volume of Earle's medium to a concentration of 1×10^5 /ml before injection of the oocyte. An aliquot of this sample was reserved for determination of both mtDNA and nDNA integrity.

Determination of mtDNA mutations by LPCR

Sperm DNA isolation. A minimum of 10^4 sperm were added to a 1.5 ml tube. This was centrifuged at 13 000 g for 3 min to pellet the cells and the supernatant removed, leaving behind 20–40 µl residual liquid. The tube was vortexed vigorously to resuspend the cells in the residual supernatant: this facilitates cell lysis. Cell lysis solution (300 µl; Flowgen, Lichfield, UK) was then added to the resuspended cells and pipetted up and down to mix thoroughly. Dithiothreitol (DTT; 200 µl of 1 M; Sigma) was then added, followed by 1.5 µl proteinase K (20 mg/ml; Sigma). This mix was inverted 25 times and incubated at 55°C overnight, until the cells had completely lysed. RNase A solution (1.5 µl; Flowgen) was added to the cell lysate and the sample inverted 25 times to aid mixing before incubation at 37°C for 60 min. The sample was cooled to room temperature (~22°C) and 100 µl of protein precipitate solution (Flowgen) added to the RNase A-treated cell lysate. This was then vortexed to mix the sample. The sample was

placed on ice for 5 min and then centrifuged at 13 000 g for 7 min. The precipitated proteins formed a tight pellet: the supernatant containing the DNA was poured into a 1.5 ml Eppendorf tube containing 300 µl isopropanol (100%; Sigma). This was centrifuged at 2000 g for 3 min prior to mixing. The supernatant was poured off and the Eppendorf tube drained on clean absorbent paper. Ethanol 300 µl (70%; Sigma), was added to the Eppendorf to wash the DNA pellet before being centrifuged at 2000 g for 3 min. The ethanol was poured off so as not to disturb the DNA. The Eppendorf tube was inverted and allowed to air dry for 15 min. Finally, DNA hydration solution (50 µl; Flowgen) were added to the DNA. This was heated at 65°C for 1 h, to rehydrate.

DNA calibration. Deionized and distilled water (490 µl) was added to a 0.5 ml quartz cuvette (Sigma) and mixed with 10 µl of the hydrated DNA sample. The DNA quantity was calculated at 260 nm using an Ultraspec II, LKB Biochrom spectrophotometer.

8.7 kb LPCR amplification. LPCR products were amplified in a Hybaid TouchDown thermal cycling system (Hybaid Ltd, Ashford, UK). LPCR using Bio-X-Act (Bioline, London, UK) was performed in 50 µl volumes. Each reaction contained 1× Optiform PCR buffer (Bioline), 0.25 mM dNTPs, 500 ng DNA template, 1.5 mM MgCl₂, 2 IU of Bio-X-Act polymerase (Bioline) and 0.5 µM of each primer (D6: 5'-TCT AGA GCC CAC TGT AAA G-3', L strand sequence, position 8286–8304; and R10: 5'-AGT GCA TAC CGC CAA AAG A-3', L strand sequence position 421–403; Lestienne *et al.*, 1997). In brief, the steps consisted of initial denaturation at 94°C for 2 min, followed by 34 cycles of denaturation at 94°C for 10 s, annealing at 52°C for 30 s and extension at 68°C for 10 min. The 'semi-hot' technique was employed, i.e. the reaction tubes, with all the components present, were placed in the thermal-cycler (Px2 thermal-cycler; Thermo Hybaid) at the start of the denaturation phase. Half (8.7 kb out of the 16.6 kb) of the mitochondrial genome was amplified from each sperm sample. This region was chosen as it encompasses the most heavily deleted region of the genome. The region amplified incorporates the following genes: complex I, the NADH dehydrogenase genes; complex III, cytochrome *b*; complex IV, the cytochrome *c* oxidase gene and CO III; and complex V, with the ATP synthase genes and up to nine of the tRNA genes (Lestienne *et al.*, 1997).

In each reaction, one negative control and one positive blood control sample were run to determine whether mis-priming of the multi-enzyme system had taken place. LPCR was repeated in duplicate samples to ensure reproducibility of the proportions of deleted mtDNA molecules and identical mutations were found. The reaction products were electrophoresed on a 0.8% agarose (Sigma) and 10× Tris-acetate–EDTA buffer (Gibco-BRL Life Technologies, Paisley, UK) gel containing ethidium bromide (1 µg/ml) at 120 V for 60 min. Electrophoresed products were photographed using Kodak gel documentation system 120 (Scientific Imaging Systems, New Haven, CT, USA).

The number of mitochondrial deletions detected was calculated as the total number of bands detected from LPCR amplification for each patient. The size of each deletion was determined by noting its position on the gel and reading its size from the ladder marker in the adjacent lane.

The mean size of the deletions was calculated using the formula: sum of all deletion sizes divided by deletion number for each patient.

Determination of DNA integrity by modified alkaline single-cell gel electrophoresis Comet assay

The following procedure (adapted from Hughes *et al.*, 1997 and Donnelly *et al.*, 1999) was performed under yellow light to prevent induced DNA damage.

Embedding of sperm in agarose gel. Fully frosted microscope slides (Richardson Supply Co. Ltd, London, UK) were gently heated, covered with 100 µl of 0.5% normal melting point agarose in Ca²⁺- and Mg²⁺-free phosphate buffered saline (Sigma) at <45°C and immediately covered with a large (22 × 50 mm) coverslip. The slides were placed in a chilled metal tray and left at 4°C for at least 30 min to allow the agarose to solidify. The coverslips were then removed and a minimum of 10⁴ sperm in 10 µl BWW were mixed with 75 µl of 0.5% low melting point agarose at 37°C. This cell suspension was rapidly pipetted on top of the first agarose layer, covered with a coverslip and allowed to solidify at room temperature.

Lysing of cells and decondensation of DNA. The coverslips were removed and the slides immersed in a Coplin jar containing freshly prepared cold lysing solution [2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris, pH 10, with 1% Triton X-100 (Sigma) added just before use] for 1 h at 4°C. Subsequently, slides were incubated for 30 min at 4°C with 10 mM DTT followed by a 90 min incubation at 20°C with 4 mM lithium diiodosalicylate (LIS; Sigma) (Robbins *et al.*, 1993).

Unwinding of DNA. Slides were removed from the lysis solution, DTT + LIS, and carefully drained of any remaining liquid. A horizontal gel electrophoresis tank was filled with fresh alkaline electrophoresis solution (300 mM NaOH, 1 mM EDTA, pH 13.0; Sigma) at 12–15°C. The slides were placed into this tank side by side, with the agarose end facing the anode, and covered with electrophoresis buffer to a level of ~0.25 cm above the slide surface. The slides were left in this high pH buffer for 20 min to allow DNA in the cells to unwind.

Separation of DNA fragments by electrophoresis. Electrophoresis was conducted for 10 min at 25 V (0.714 V/cm) adjusted to 300 mA by raising or lowering the buffer level in the tank. After electrophoresis the slides were drained, placed on a tray and flooded with three changes of neutralization buffer (0.4 M Tris, pH 7.5; Sigma) for 5 min each. This removed any remaining alkali and detergents that would interfere with ethidium bromide staining. The slides were then drained and stained with 50 µl of 20 µg/ml ethidium bromide (Sigma) and covered with a large coverslip. The slides were viewed using a Nikon E600 epifluorescence microscope that was equipped with an excitation filter of 515–560 nm from a 100 W mercury lamp and a barrier filter of 590 nm. Fifty images were captured and analysed by an image analysis system using the programme Komet 3.1 (Kinetic Imaging Ltd, Liverpool, UK).

Statistical analysis

Mitochondrial and nDNA results were analysed using SPSS version 11.0 (www.SPSS.com). Student's *t*-test for independent samples was employed to determine the differences between testicular or ejaculated samples for successful and unsuccessful cycles. Logistic regression was used to determine the relationships between nDNA and mtDNA.

Initially, two separate databases were set up. One looked at the effects of using ejaculated sperm on assisted conception outcome. The second looked at the effects of using testicular sperm on assisted conception outcome. It was questionable whether the two databases could be combined. This was tested by adding an explanatory dichotomous variable to identify the source of the sample. Sperm source was not a significant factor in this model, indicating that source is only important insofar as better quality sperm (see independent samples *t*-tests presented in Table I) tends to be obtained from testicular sperm, and consequently the combination of sources increased the range of each of the sperm quality variables. Once these factors were taken into consideration there was no further contribution from the source of sperm. Three sperm quality variables were thought to be related to a positive pregnancy outcome. These

Figure 1. The relationship between fertilization rates and mtDNA deletion number and size ($n = 68$), the regression coefficient between mtDNA number and fertilization is $r = -0.05$ ($P > 0.05$). The regression coefficient between mtDNA size and fertilization is $r = -0.17$ ($P > 0.05$).

Table I. The relationship between nDNA, mtDNA and ICSI outcome					
Sperm source	<i>n</i>	ICSI/TICSI outcome	nDNA fragmentation (%)	mtDNA deletion number	mtDNA deletion size (kb)
Testicular	14	Pregnant	16.1 ± 1.7	1.29 ± 0.29	3.08 ± 0.52
Testicular	14	Not pregnant	26.4 ± 3.1*	2.86 ± 0.35*	5.91 ± 0.41*
Ejaculated	26	Pregnant	21.4 ± 2.1†	2.43 ± 0.29†	3.90 ± 0.30†
Ejaculated	41	Not pregnant	27.4 ± 1.5*	3.29 ± 0.22*	4.69 ± 0.27*

Values are means ± SE.
* $P < 0.05$ significant differences between successful and unsuccessful outcome with testicular or ejaculated sperm.
† $P < 0.05$ significant differences between testicular and ejaculated sperm.

were nDNA fragmentation, the number of mtDNA deletions and the size of the deletions (if present).
The outcome (pregnancy/no pregnancy) is also dichotomous, so logistic regression was run with the four explanatory variables as described above. Deletion size is obviously dependent on number of deletions, even if the extent of this relationship is only that no size can be determined if deletion number is 0. No correlation was found between deletion size and deletion number where both variables were measured (i.e. deletion number at least 1). If deletion number and deletion size are both important, it would be unfortunate to lose those cases in the analysis where deletion number was 0, simply because of no information on deletion size. Since there was no observed correlation between the two variables, a mean deletion size (of 4.3 kb) was included in cases where the deletion number was 0.
The result (see Table II) was a logistic regression model with nDNA fragmentation, mitochondrial deletion number and deletion size as independently significant factors in the odds of a successful outcome.
The key outcome from the model derived above is individual posterior probabilities of a positive pregnancy (see Figure 1) We tested the performance of our prognostic model by calculating the c-statistic, which is identical to the area under the receiver operator characteristic (ROC) curve. Essentially, all possible pairs of individuals where one is pregnant and one is not pregnant were considered. Then, the number of such pairs where the posterior probability for the pregnant couple is higher than the posterior probability for the non-pregnant couple was counted. The latter, a ratio of the former, was defined as the c-statistic. A null performance of the model would result in a c-statistic of ~0.5. Our actual value was 0.83, indicating its strong prognostic ability.
The logistic regression model provides posterior probabilities of pregnancy as final output. Whilst these probabilities are useful to

Table II. Modelling the likelihood of successful outcome by logistic regression						
Parameter	B	SE	Wald	df	Exp(B)	<i>P</i>
ICSI versus TICSI	-0.11	0.61	0.031	1	0.898	0.860
nDNA fragmentation	-0.07	0.03	5.730	1	1.068	0.017
mtDNA deletion no.	-0.53	0.18	8.385	1	0.586	0.004
mtDNA deletion size	-0.46	0.16	8.374	1	0.633	0.004

The table shows that nuclear DNA fragmentation and mitochondrial deletion number and size are independently significant in predicting outcome. Furthermore, the source of sperm is not important over and above these three parameters.

statisticians, they are not particularly user friendly to clinicians—not least because a computer program, incorporating logistic regression predictions, will not always be available in the clinic. We have further explored the utility of our model by deriving a simpler prognostic score, based on the magnitude of the coefficients in the finally selected logistic model. To check the validity of the scoring system, the scores were regressed against the probit transformation of the posterior probabilities. The probit transformation also normalizes the posterior probabilities (see Figure 1), which is advantageous when there is some skew in the data. For instance, a model that is asked to discriminate between a large number of ‘touch and go’ cases has a much harder task than one asked to discriminate between a distribution of cases in which posterior probabilities close to 0 or 1 occur frequently. We have constrained these scores in such a way so that a nominal score of <80 represents a very high chance of pregnancy being achieved in this treatment cycle (see algorithm).

Results
Outcome of ICSI cycles

From the 28 couples undergoing TICSI 14 couples became pregnant (50%). From the 77 couples starting TISCI cycles, 66 couples had embryo transfers and 26% of these couples became pregnant (39%).

Relationship between the number and size of sperm mtDNA deletions and fertilization rates

There is no relationship between the number or size of mtDNA deletions in either testicular or ejaculated sperm and fertilization rates in ICSI. The data from each group are combined in

Figure 2, showing that neither deletion number ($r = 0.05$) nor size ($r = 0.17$) has a significant relationship with fertilization rates.

The relationship between sperm nDNA fragmentation and ICSI outcome with testicular and ejaculated sperm

There was no significant relationship between nDNA fragmentation and fertilization rates (data not shown). Nuclear DNA was more fragmented ($P < 0.01$) in both testicular and ejaculated sperm of men whose partners did not become pregnant (Table I). There was also significantly more DNA fragmentation in ejaculated sperm compared with testicular sperm (Table I).

The relationships between mtDNA deletion number, size and ICSI outcome for testicular and ejaculated sperm

There were a significantly higher number of mtDNA deletions in both testicular and ejaculated sperm of men whose partners did not become pregnant (Table I). These deletions were also of a larger scale (Table I). There were also significantly more mtDNA deletions of larger size in ejaculated sperm compared with testicular sperm (Table I).

Comparison of the incidence of mtDNA deletions in testicular sperm in successful and unsuccessful TICS I cycles

Of the patients who became pregnant, only 26% had partners with wild type (WT) sperm mtDNA. Single deletions were observed in 37% of partner's sperm, double deletions in 18.5% and multiple (i.e. more than two deletions) in the remaining 18.5% of the group. In contrast, in those patients who did not become pregnant, only 6.5% had partners with WT sperm mtDNA and a much higher proportion (65%) had multiple deletions (Figure 3).

The comparison in incidence of mtDNA deletions in ejaculated sperm in successful and unsuccessful ICSI cycles

Only 8% of patients who became pregnant and 2% of patients who did not become pregnant had partners with WT sperm mtDNA. Single deletions were observed in 16% of partner's sperm, double deletions in 13% and multiple (i.e. more than two deletions) in the remaining 62% of the group who became pregnant. Similarly, in those patients who did not become pregnant, single deletions were observed in 9% of partner's sperm, double deletions in 11% and multiple (i.e. more than two deletions) in the remaining 77% (Figure 4).

The relationship between mtDNA deletion number and size and nDNA fragmentation

There was a significant relationship between mtDNA deletion number and nDNA fragmentation ($r = 0.32$, $P = 0.004$) (Figure 5A). In contrast, no significant relationship was observed between mtDNA deletion size and nDNA fragmentation ($r = 0.19$, $P = 0.11$) (Figure 5B).

Figure 2. The comparative incidence of single, double and multiple mtDNA deletions in testicular sperm in successful and unsuccessful ICSI cycles. Black bars: successful cycles [$n = 10$; 26% of subjects displayed wild type (WT)]. Grey bars: unsuccessful cycles ($n = 18$; 6% of subjects displayed WT). All the mtDNA fragments add up to 100%.

Figure 3. The comparative incidence of single, double and multiple mtDNA deletions in ejaculated sperm in successful and unsuccessful ICSI cycles. Black bars: successful cycles ($n = 28$; 8% displayed WT). Grey bars: unsuccessful cycles ($n = 49$; 2% displayed WT). All the mtDNA fragments add up to 100%.

The combination of nDNA and mtDNA damage to provide a prognostic test

Since it is rarely desirable for a clinician to feed data into a logistic regression 'black box' to produce a posterior probability, we have devised a simple scoring system that has a strong relationship with the probability of a positive outcome (see Appendix for algorithm).

Discussion

This the first time that an assessment of the both nuclear and mitochondrial DNA of sperm has been used to predict the pregnancy rate in assisted conception by ICSI with either ejaculated or testicular sperm. Although ICSI is a major advance in male infertility treatment, concerns remain about possible long-term deleterious effects. ICSI circumvents all the natural barriers to fertilization (adequate concentration, motility and morphology), thus facilitating the union of potentially defective gametes. There are reports that children

Figure 4. The relationship between nDNA fragmentation and mtDNA deletion number and size ($n = 80$). The regression coefficient between nDNA fragmentation and mtDNA deletion number was $r = 0.32$ ($P = 0.004$) ($n = 70$). The regression coefficient between nDNA fragmentation and mtDNA mean deletion size was $r = 0.19$ ($P = 0.11$).

Figure 5. Graphical description of probability. A plot of prognostic score against predicted probabilities from the regression model showing linearity over a broad range of probabilities. For example, a prognostic score of 84 gives a probability of pregnancy of 91%, a prognostic score of 47 gives a probability of pregnancy of 47% and a prognostic score of 3 gives a probability of pregnancy of only 5%.

born through ICSI have a higher incidence of de-novo chromosomal abnormalities (Foresta *et al.*, 1996; te Velde *et al.*, 1998), and there are also data to suggest that the mental development of ICSI babies is slower (Bowen *et al.*, 1998), although this has not been confirmed in a more recent study (Bonduelle *et al.*, 2000). Yet, rather than proceeding with caution, the application of ICSI has rapidly widened so that even injection of immature testicular sperm is now routine.

Traditionally, pregnancy rates have been associated primarily with the female partner. However, since ICSI facilitates fertilization by sperm that nature would reject, it is important to assess the quality of paternal genetic material and to establish criteria by which to choose appropriate cohorts of sperm. Assisted reproduction is expensive financially and emotionally, highly invasive and the long-term consequences remain

unknown; therefore, couples justifiably want to know the likelihood of success before embarking on a treatment cycle. These patients have a right to the fullest information that we can provide. Although this was a retrospective analysis with relatively small numbers, we believe we have a test that would appear to give the couple and their clinician this information. We suggest that both nuclear and mitochondrial DNA quality of the sperm should be assessed before their cohort is used clinically, thereby giving the couple an accurate prediction of the likelihood of pregnancy with that sample.

This is also the first study, to our knowledge, to investigate a close inverse relationship between the testicular sperm pregnancy rates in ICSI, and the level of DNA fragmentation. In agreement, we observed a similar significant negative correlation for ejaculated sperm. This confirms the view that a sperm nDNA fragmentation test is a valuable addition to current prognostic tests, irrespective of the sperm source and the sperm's level of maturity. We have shown that testicular sperm head size is substantially larger than that of ejaculated sperm (Steele *et al.*, 2000). By Tygerberg criteria, testicular sperm heads would be classified as large and amorphous and, in addition, they often have a cone shape of cytoplasm around the midpiece. Rather than being abnormal, we believe that this simply indicates a lack of maturity from incomplete chromatin compaction. The protamination that gives sperm DNA such protection is not completed until S-S bonds form between protamine molecules during epididymal transit. Thus, it is interesting to note that DNA fragmentation is also prognostic of ART success in these immature sperm. Our data support those of Sun *et al.* (1997) and Ahmadi and Ng (1999), who found a negative association between embryo cleavage rates and DNA fragmentation measured by the TUNEL assay. They are also in agreement with data obtained using the sperm chromatin structure assay, where an increased susceptibility to nDNA denaturation reduced the chances of a pregnancy following ART (Evenson *et al.*, 1999). In first-pregnancy planners, with no knowledge of their fertility capability, time to pregnancy increased as a function of the proportion of sperm with abnormal chromatin (Spano *et al.*, 2000). Evenson *et al.* (1999) have further showed that the miscarriage rate was

higher in apparently fertile couples where the partner's sperm had poor chromatin quality. Furthermore, assessment of nuclear packaging by ethidium bromide binding (Filatov *et al.*, 1999) indicated that abnormalities in chromatin packing reduced rates of embryo cleavage, again showing a link between sperm DNA and male fertility potential. For this reason, Sakkas *et al.* (2000) have suggested the use of blastocyst culture to avoid the inheritance of an abnormal paternal genome after ICSI. Extended culture will give a test by which to choose only viable embryos, which may well reflect the quality of the gametes from which they were derived (Behr *et al.*, 1999).

Using sperm with damaged DNA may have far-reaching consequences on the health of offspring. One example of this is that fathers with DNA-damaged sperm due to smoking are more likely to have children with cancer (Sorahan *et al.*, 1997). Animal studies have shown that damaged DNA can lead to increased post-implantation loss and are transmissible to the next generation (Hales *et al.*, 1992). Other concerns include indications that intracellular signalling is modified with the ICSI procedure (Tesarik *et al.*, 1994), and that first mitotic divisions may be disturbed (Hewitson *et al.*, 1999). Furthermore, it has been postulated that ICSI with surgically retrieved sperm could interfere with the genomic imprinting of the sperm genome (Tycko *et al.*, 1997).

The high level of DNA fragmentation observed in these studies may be due to environmental toxins and/or oxidative damage. As already mentioned, smoking leads to an increase in fragmented sperm DNA (Sun *et al.*, 1997). Increased exposure to environmental estrogen or 'endocrine disrupters' has also been shown to produce developmental, structural, functional and teratogenic changes in the male genital tract (Sharpe, 1998). Various types of DNA damage have been found to be caused by exogenous and endogenous estrogens (Liehl, 2001). Our group has also observed DNA damage in sperm exposed to high levels of dietary phytoestrogens (unpublished observations). Another culprit leading to DNA damage is oxidative stress. Sperm are particularly susceptible to damage from reactive oxygen species (ROS) due to their unsaturated fatty acid membranes and lack of repair mechanisms. Many studies have reported a connection between such stress and DNA damage (Cummins *et al.*, 1994; Twigg *et al.*, 1998; Donnelly *et al.*, 1999). At high levels of oxidative stress, multiple sperm functions are compromised (Aitken *et al.*, 1998). The number of patients (26%) in this study with WT mtDNA is lower than in some of our previous studies (~45%) (O'Connell *et al.*, 2002). One explanation for this may lie in the differing aetiologies of the obstruction. Obstructive azoospermia may occur for a number of reasons. The most common causes are vasectomy, coincidental ligation or resection of the vas during scrotal or hernia surgery, infection (gonorrhoea, chlamydia or tuberculosis) or congenital bilateral absence of the vas deferens (CBAVD). The degree of compromise of testicular sperm mtDNA in these infertile men may depend on the cause of the blockage. mtDNA may be affected by a range of conditions, from temperature fluctuations to transit times, as well as exposure to hydrolysing enzymes such as hyaluronidase and acrosin from dead and dying sperm acrosomes (Cummins *et al.*,

1994). Depending on the site of the obstruction, testicular sperm may be damaged by oxidative stresses within the testis caused by stasis or from a build-up of localized pressure resulting from the obstruction (Hess, 1998). It is also possible that in these patients, this increased pressure delays the release of sperm from the seminiferous tubules, leading to premature ageing of the sperm, the effects of which are displayed as accumulated mtDNA deletions. Another example is in patients whose obstruction is caused by infection with its associated temperature increase. They may experience a greater deterioration in their sperm mtDNA quality than those with CBAVD from birth. Further studies are required to relate mtDNA status to aetiology and, in the case of vasectomy, the length of time since the surgically induced obstruction.

These are the first data to study associations between mtDNA and assisted conception outcome. As with nDNA, we observed no relationship between the number or size of mtDNA mutations of either testicular or ejaculated sperm and fertilization rates in TICS. In contrast, in those patients who became pregnant following ICSI, their partners had significantly higher levels of non-mutated (WT) sperm mtDNA and a much lower proportion of multiple deletions. This was true of both testicular and ejaculated sperm, although the association was stronger in the former. The data suggest that the quality of mtDNA, as well as nDNA, is a good prognostic factor for successful outcome. The quality of sperm mtDNA is important for two reasons. Sperm metabolism is dependent, at least in part, on energy derived from oxidative phosphorylation in the inner mitochondrial membrane (Wallace *et al.*, 1994). If the mitochondria are dysfunctional, they may be incapable of providing sufficient ATP for optimal motility. In this study we probed the mitochondrial genome in the region located between the D loop and Cox II gene. This region contains the complexes involved in oxidative phosphorylation such as cytochrome *c* oxidase, ATPase, NADH dehydrogenase, cytochrome *b* and up to nine tRNAs (Lestienne *et al.*, 1997). Thus, large or multiple deletions here may indicate major disruption to the electron transport chain (ETC), which would reduce ATP output and impair metabolism.

Functional membrane potentials are important to mitochondria and are achieved by the pumping of protons across the inner mitochondrial membrane as a result of oxidative phosphorylation (Donnelly *et al.*, 2000). However, the mitochondrial respiratory system is also the major intracellular source of ROS. These are generated as by-products during the transfer of electrons in the ETC and complexes to molecular oxygen within the inner mitochondrial membrane. This increase in ROS may well result in an increase in mtDNA deletions. The most immediate effect of oxidative assault will be observed in the mitochondria, since the matrix of the mitochondrion is in closest proximity with ROS. Sperm mtDNA and nDNA are particularly vulnerable to damage induced by endogenous ROS due to the cell's absence of significant repair mechanisms and high content of membrane polyunsaturated fatty acids. This exposure to ROS can progress to plasma membrane damage through an accumulation of lipid peroxides (Selley *et al.*, 1991). The production of excessive ROS, due to either increased generation or reduced antioxidant

protection (Lewis *et al.*, 1995), is now thought to underlie many aspects of human male infertility, where sperm are rendered dysfunctional by lipid peroxidation and altered membrane function, together with impaired metabolism and motility (Cummins *et al.*, 1994).

The proportion of patients with WT sperm mtDNA in ejaculated sperm was extremely low in both those who did (8%) and those who did not (2%) achieve a pregnancy. The majority of partners' sperm had multiple deletions. It is surprising to see such a high incidence of deletions—much higher than those commonly observed in somatic cells (Lestienne, 1992). It may be that in the evolution of the species, if paternal mtDNA is to be expelled from the embryo at an early stage (Sutovsky *et al.*, 1999), some of the mechanisms that are present in the oocyte to protect mtDNA have become obsolete during sperm differentiation (Reynier *et al.*, 1998), allowing mtDNA mutations to accumulate in sperm. However, the importance of non-mutated mtDNA in sperm cannot be disregarded. Recent findings by St John *et al.* (2000) and St John and DeJonge (2000) contrast with Sutovsky's study (Sutovsky *et al.*, 1999), and provide compelling evidence for the persistence of paternal mtDNA well beyond the 8-cell stage and into the blastocyst stage of normal and abnormal embryos created by IVF and ICSI techniques. While it is reassuring to note from the studies of Torroni *et al.* (1998) and Danan *et al.* (1999) that the ICSI procedure did not alter the uniparental pattern of mtDNA inheritance, these studies were performed on children conceived using mature, i.e. ejaculated, sperm. As yet there are no studies to confirm that mechanisms for paternal mtDNA expulsion are not compromised when immature sperm are used, and this is another reason why characterization of mtDNA used for clinical purposes is important.

To our knowledge, we are the first group to demonstrate a relationship between nDNA fragmentation and the number of mtDNA deletions in both testicular and ejaculated sperm. In somatic cells there is a bi-directional flow of information between nucleus and mitochondrion. However, in somatic cells there are no physical barriers between the cytoplasm and the nucleus and the genomes interact in the synthesis and assembly of mitochondrial proteins, particularly for oxidative phosphorylation (Poyton and McEwen, 1996). In contrast, in post-spermiogenic sperm, the nuclear genome is largely inactive and the nuclear and mitochondrial compartments are physically isolated by the cytoskeleton, making exchange of information much more difficult. Such cross-talk has been shown to be unimportant for metabolic pathways in the head, midpiece and flagellar compartments. The relationship between mtDNA deletions and nDNA fragmentation in sperm suggests that some interaction may still exist between the genomes, in that malfunction of one is observed in the other. Alternatively, the assault on both mtDNA and nDNA may be so great that both respond similarly to their environment despite differences in how each genome is protected, because in each the defence is inadequate. The most probable mechanism to explain this correlation is that DNA damage in sperm is caused, to a great extent, by ROS, gonadotoxicants and/or xenobiotics.

In contrast to the relationship with pregnancy rates, we found no relationship between DNA fragmentation and fertilization rates in ICSI using ejaculated or testicular sperm. These results are in agreement with others (Twigg *et al.*, 1998; Host *et al.*, 2000) who showed that damaged DNA did not prevent fertilization or pronucleus formation (Twigg *et al.*, 1998) after ICSI, suggesting that the functional competence of the sperm is not essential for these processes. This may be expected, since the nature of the ICSI technique facilitates fertilization without the processes of capacitation, acrosome reaction or penetration of the zona pellucida.

It has also been suggested that the paternal genome does not exert its influence until around the 8-cell stage of the embryo (Braude *et al.*, 1988), although a recent study has shown a paternal influence in the first cell cycle after ICSI (Tesarik *et al.*, 2002). In addition, the maintenance, and indeed survival, of somatic cells depends on their DNA integrity, although sperm appear to function without significant nuclear control. Even in IVF, where sperm competence is a prerequisite, there is conflicting evidence about the relationship between sperm nDNA fragmentation and fertilization rates. The presence of damaged DNA (regardless of the degree of damage) was reported to have no effect on fertilization in one study (Ahmadi and Ng, 1999), but was shown to have a significant inverse relationship in others (Sun *et al.*, 1997; Host *et al.*, 2000) and to contribute to a failure of fertilization even in ICSI in a fourth study (Lopes *et al.*, 1998).

In conclusion, this study suggests that combining data about DNA quality from both sources within sperm provides a potentially valuable prognostic test. The information has been incorporated into a simple scoring system for clinicians to use to counsel patients more appropriately. For the first time, by using this algorithm, the clinician may be able to give couples a more quantitative estimate of their chances of a pregnancy from their treatment. A large prospective study is now ongoing to test the validity of the algorithm.

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Appendix

Algorithm for the determination of treatment outcome by sperm DNA quality

The calculation is based on the nuclear DNA fragmentation (%), the mitochondrial deletion number and, where deletion number is positive, the deletion size. The higher the values the less probable it is that a pregnancy will occur.

Determination of score

1. Nuclear component

Correct the nuclear DNA fragmentation to the nearest percent and subtract from 90 = (A)

2. Mitochondrial component

(a) Deletion number

(i) If the mitochondrial deletion number is 0, (A) is your final value

(ii) If the deletion number is not 0, remove 8 for each deletion = (B)

(b) Deletion size

(i) If mean deletion size is less than 4, subtract the value from 4 and multiply by 7 = (C)

(ii) If mean deletion size is greater than 4, subtract 4 from the value and multiply by 7 = (C)

3. The final score is (A) + (B) + (C) = (D)

If the score is <20, the couple has a low chance of pregnancy and should be counselled accordingly. As the score increases, the couples' chance of a pregnancy increases proportionately. If the score is >80, there is a very high probability that a pregnancy will ensue.