Article

Comet assay of cumulus cells and spermatozoa DNA status, and the relationship to oocyte fertilization and embryo quality following ICSI

Born in Saudi Arabia, Ms Abu-Hassan was educated at an international American school. Moving to Egypt, she studied for A-level examinations at the Modern English School in Cairo. She went on to study at the American University in Cairo. She was awarded the Bachelor's degree in June 2002, majoring in biology with chemistry as a minor subject; she graduated with high honours. Then she joined the Department of Assisted Reproduction in Lübeck University, Germany, under the supervision of Professor Safaa Al-Hasani, where she became proficient in various assisted reproduction techniques and was involved in research. She now works back in Egypt in the fertility laboratory of a private hospital.

Ms D Abu-Hassan

D Abu-Hassan, F Koester, B Shoepper, A Schultze-Mosgau, B Asimakopoulos, K Diedrich, S Al-Hasani[1]
Department of Assisted Reproduction, Women's Hospital, Medical University of Schleswig-Holstein, Ratzeburger Allee 160, 23538 Lübeck, Germany
[1]Correspondence: e-mail. Sf_alhasani@hotmail.com

Abstract

It has been postulated that apoptosis may affect cumulus cell and sperm DNA integrity, and therefore influence the outcome of assisted reproductive techniques. This study investigates apoptotic levels in both cumulus cells and spermatozoa, and their relationship with fertilization and embryo quality after intracytoplasmic sperm injection (ICSI). The neutral comet assay was performed on cumulus cells and semen samples from 55 couples with male factor infertility undergoing ICSI treatment. Cells were fixed in agarose on comet assay slides, lysed in a neutral buffer and submitted to electrophoresis. The cells were stained with SYBR green fluorescent dye, which binds to double-stranded DNA and upon excitation emits light. Analysis showed that there was no correlation between apoptosis levels and the outcome of ICSI (fertilization and embryo quality).

Keywords: *comet assay, DNA fragmentation, embryo quality, ICSI results*

Introduction

Male factor infertility accounts for approximately 50% of all cases attending infertility clinics (Morris *et al.*, 2002). Traditional semen analysis is based on sperm concentration, motility and morphology (Tomlinson *et al.*, 2001; Morris *et al.*, 2002). With the advancement of assisted reproductive techniques and especially the use of intracytoplasmic sperm injection (ICSI), the requirements of semen analysis are changing and becoming more specific and more informative (Tomlinson *et al.*, 2001). Since ICSI removes many of the natural selection barriers to ensure selection of the best spermatozoa for injection, it is important to have strict and useful criteria in order to select these spermatozoa, the most important criterion being DNA integrity. There is evidence to suggest that sperm DNA integrity does affect fertilization, preimplantation embryo development, pregnancy and fetal abnormalities (Morris *et al.*, 2002; Tomsu *et al.* 2002). However, analyses do not consider male genome integrity and so the 'best' spermatozoa are not chosen by DNA integrity, but rather by morphology and motility (Morris *et al.*, 2002), which have no relation with genetic condition (Bianche *et al.*, 1996). It would therefore be useful to be able to analyse sperm DNA and include it in semen analysis prior to treatment, and select spermatozoa for ICSI based on the DNA status.

The causes of male infertility may be due to mutation or deletion of genetic information in the sperm genome, but it may also be due to other genetic conditions, including DNA fragmentation due to necrosis or apoptosis (Morris *et al.*, 2002; Sakkas *et al.*, 2003).

Apoptosis (programmed cell death) is characterized by double-stranded DNA breaks, resulting in regular DNA fragments with lengths in multiples of approximately 180 base pairs (Olive

and Banath, 1995). The general theory of apoptosis is that it is a mechanism for controlling cell populations by eliminating excessive and defective cells (Ricci *et al.*, 2002), and it occurs in somatic- and germ-cell populations. This theory is supported by the estimation that one spermatogonia in the human testes gives rise to approximately 100 spermatids, and not to 4096, the theoretical value (Shen *et al.*, 2002).

In ovarian follicles, cumulus cells are closely related to the oocyte forming the oocyte–cumulus complex. The cumulus cells are shed and undergo apoptosis following ovulation, and therefore there is a hypothesis that the oocyte maturity can be determined through the DNA status of the cumulus cells that are in close association with the oocyte (Raman *et al.,* 2001), and consequently be related to fertilization. Recent studies have further elucidated the relationship of cumulus cells, in the context of gene expression for PTGS2 (cyclooxygenase 2), HAS2 (hyaluronic acid synthase 2) and GREM1 (gremlin), with the oocyte quality, fertilization rates and embryo quality (McKenzie *et al.*, 2004)

During natural folliculogenesis, one follicle becomes the dominant follicle, while the others undergo atresia. In ICSI all the follicles are punctured with no knowledge of whether the oocyte inside is mature or not. Therefore, the apoptotic levels of these cumulus cells is of interest, to try and determine if indeed there is a relation between apoptosis and the maturity of oocytes, and thereby the selection of the dominant follicle in natural cycles, and the selection of suitable oocytes for injection with ICSI.

In the case of spermatozoa, two opposing hypotheses have been suggested, regarding the relationship between apoptosis levels and fertilization. Increased levels of apoptosis indicate, or are positively related to, decreased fertilization rates (Saleh *et al.*, 2002; Moustafa *et al.*, 2002), while Morris *et al.* hold the opposing view that increased apoptotic levels are related to increased fertilization due to the elimination of defective spermatozoa.

The comet assay, which uses single cell gel electrophoresis (SCGE) to analyse DNA fragmentation in individual cells, was first introduced in 1984 by Ostling and Johanson (Ostling and Johanson, 1984; Olive and Banath,1995), who used neutral buffer conditions to study double-stranded DNA breaks(Raman *et al.,* 2001). It was later modified using alkaline electrophoresis buffers to increase the sensitivity to both single and double-stranded DNA breaks, by Singh and colleagues (Raman *et al.,* 2001). The comet assay may therefore be used to study single or double-stranded DNA breaks in somatic cells or in germ cells, and is useful because it allows for the distinction between the different kinds of DNA fragmentation, e.g. necrotic versus apoptotic. Apoptotic cells produce teardrop-shaped comets (Olive and Banath, 1995) during electrophoresis. The shape is due to the migration and accumulation of the short DNA fragments, and the intensity of the tail represents the amount of DNA fragments present (Duty *et al.*, 2002). In this case, neutral conditions were used since it is a study of apoptotic DNA fragmentation, which is characterized by double-stranded DNA breaks.

Taking all this into consideration, it was thought interesting to investigate if there is a relation between apoptosis in spermatozoa and cumulus cells, and the outcome of ICSI; fertilization and embryo quality. A comparison was also carried out between apoptotic levels in native spermatozoa and spermatozoa processed by the swim-up method in preparation for ICSI.

Materials and methods

Patient selection

Couples undergoing ICSI treatment at the Department of Assisted Reproduction in the University of Luebeck were used in this study. There was no age limit on either partner and couples did not have to be childless, as is required in other studies (Morris *et al.*, 2002). The study included couples with no genetic disorders (patients who underwent polar body biopsies were excluded) as well as patients who provided ejaculated semen (testicular sperm extraction and micro-epididymal sperm aspiration patients were excluded). According to the patients' detailed medical historys, clinical examinations and laboratory findings, no other major causes of male infertility were detected except for smoking.

ICSI treatment cycles

Ovarian stimulation was achieved in over 75% of the patients using the short protocol, gonadotrophin-releasing hormone (GnRH) antagonist and recombinant FSH.

Oocytes were recovered by ultrasound-guided follicular puncture. Follicles were punctured and drained one by one until four individual oocytes were obtained. Oocytes were denuded, the cumulus cells washed in phosphate-buffered saline (PBS) and hyaluronidase added to disperse the cells. Each of the four individual cumuli was washed alone and kept in a 1.5 ml Eppendorf tube, while the cumuli of any other oocytes collected were pooled together.

Semen samples were produced by masturbation and analysed for sperm concentration, motility and morphology. They were then prepared by the swim-up method and incubated. For the purpose of this research two semen samples were used. The comet assay was performed on a sample of washed, native semen and on a sample of the 'swim-up' used for ICSI. ICSI was performed using standard protocols (Al-Hasani *et al.,* 1995).

Fertilization and embryo development

On day 1 (16–18 h after insemination), oocytes were assessed for pronuclei presence. According to German law, a maximum of three embryos can be transferred to the uterus, and only oocytes in the pronuclei stage may be frozen or discarded, therefore the number of embryos desired by the couple is the number of normally fertilized oocytes cultured to cleave to the 2- or 4-cell stage (day 2). If cryopreservation was asked for, excess zygotes (in the PN stage) were stored for future use, and if no cryopreservation was asked for, the excess zygotes were discarded.

On day 2, a maximum of three embryos were introduced into the uterus. Embryos were graded based on the grading systems

of Veeck (1999): G1, ideal (no fragmentation and regular blastomeres); G2, good (minor-to-moderate fragmentation and irregular blastomeres); and G3, irregular (significant-to-severe fragmentation and unequally sized blastomeres). Embryo score was then calculated in order to provide a numerical value that could be subjected to statistical inquiry. Each embryo grade was assigned a value (G1 = 3, G2 = 2, G3 = 1) and the embryo score was the product of this value and the number of blastomeres in the embryo.

COMET assay

Cumulus cells

Cells were cast in agarose on comet assay slides (each slide holds two gels) obtained from Trevigen (Maryland, USA) and refrigerated. They were then lysed (at 4ºC for minimum 30 min) to degrade the membranes, release DNA associated proteins and allow the DNA to unwind. Lysis buffer (2.5 mol/l NaCl, 10 mmol/l Tris base and 100 mmol/l EDTA, pH 10; TBE) contained 1% lauryl-sarcosine and 1% Triton X-100. The gels were then washed in 1× TBE and electrophoresed (10 min at 14 V) in neutral buffer (1× TBE). Damaged, negatively charged DNA migrated from the nucleus towards the anode. The slides were then dried in 70% ethanol, and once dry stained with SYBR Green DNA-binding fluorescent dye (BMA BioWhittaker Molecular Application, Rockland, ME, USA), viewed using a Zeiss Axiovert 135 epifluorescence microscope, Zeiss, Jena, Germany.

No measurements of comet tail were performed. The slides were viewed under ×40 magnification, the number of apoptotic comets counted (characterized by tear-shaped comets) and the percentage of apoptotic comets calculated.

Spermatozoa

The procedure was similar to that used for cumulus cells. If necessary, samples were diluted using 1× PBS (without Ca^{2+} and Mg^{2+}). Native sample was then cast into gel 1 and processed sample in gel 2 on one comet assay slide. The main difference to the cumulus cell procedure was the lysis stage. Different buffers were used, the sperm lysis buffer provided by Dr MH Nasr-Esfahani (Royan Institute, Tehran, Iran). Lysis was initially carried out for 1 h. The procedure was later modified, and lysis carried out overnight, due to the extremely tight nature of sperm–DNA compaction. Gels underwent electrophoresis for 15 min at 15 volts, dried with 70% ethanol and viewed like the cumulus slides.

Statistical analysis

Analysis was carried out by dividing the data into groups based on fertilization (0PN, 2PN, 3PN). Comparisons between the groups were each performed twice using both the Mann–Whitney *U*-test and the Kolmogorov–Smirnov test. The Spearman rank order correlation coefficient was used for all the correlation studies.

Results

The percentage of apoptosis in both sperm and cumulus cell (CC) populations from 55 couples was determined by the neutral comet assay. In order to analyse the data obtained, it was divided into three groups based on the number of pronuclei present: PN0 group, PN2 group and PN3 group.

Tables 1 and **2** present the summary data of the comparisons performed between the PN0 and PN2 groups, and the PN2 and PN3 groups respectively. No statistically significant difference was found between the ages of the females of the compared groups, and accordingly there was no correlation found between age and the percentage of apoptosis (**Table 3**). There was also no significant difference between the groups of both pairs in the percentage of cumulus cell apoptosis (CC%AP), and the percentage of apoptosis in both the native (N%AP) and processed (P%AP) semen samples. The only significant difference was found to be the embryo score (between PN0 and PN2 the embryo score *P*-level was ≤0.01 and between PN2 and PN3 the embryo score *P*-level was ≤0.05).

Correlation studies showed that there was no correlation, however, between age and embryo score or between embryo score and percentage of apoptosis (**Table 4**).

Table 1. Comparisons of apoptosis in sperm and cumulus cell populations according to the number of pronuclei: PN0 and PN2 CC = cumulus cell; AP = apoptosis; ES = embryo score, N = native spermatozoa, P = processed spermatozoa.

Variable	*Mean PN0*	*SD PN0*	*Mean PN2*	*SD PN2*
Maternal age (years)	33.03	3.45	33.14	3.77
CC%AP	2.716	3.150	2.965	2.942
ES	0.121[a]	0.985	6.726[a]	4.335
N%AP	0.559	1.064	0.849	1.814
P%AP	0.675	1.582	0.503	1.098

[a]Significantly different ($P < 0.01$).
There were no other significant differences.

Table 2. Comparisons of apoptosis in sperm and cumulus cell populations according to the number of pronuclei: PN2 and PN3. CC = cumulus cell; AP = apoptosis; ES = embryo score; N = native spermatozoa; P = processed spermatozoa.

Variable	*Mean PN0*	*SD PN0*	*Mean PN2*	*SD PN2*
Maternal age (years)	33.14	3.77	32.44	3.54
CC%AP	2.965	2.942	2.366	2.082
ES	6.726[a]	4.335	2.444[a]	3.972
N%AP	0.849	1.811	0.244	0.236
P%AP	0.503	1.098	0.000	0.000

[a]Significantly different ($P < 0.05$).
There were no other significant differences.

Table 3. Correlations in total cases (PN0, PN2 and PN3) with maternal age. CC = cumulus cell; AP = apoptosis; ES = embryo score; N = native spermatozoa; P = processed spermatozoa. There were no statistically significant correlations.

Pair of variables	*Valid* n	*Spearman* R	t(n–2)
Age and CC%AP	187	0.77413	1.05617
Age and ES	188	0.003004	0.04097
Age and N%AP	49	–0.253077	–1.79339
Age and P%AP	51	–0.036653	–0.25674

Table 4. Correlations between embryo score and percentage apoptosis. CC = cumulus cell; AP = apoptosis; ES = embryo score; N = native spermatozoa; P = processed spermatozoa. There were no statistically significant correlations.

Fertilization Group	*Pair of variables*	*Spearman* R	t(n–2)
PN0	ES and CC%AP	–0.173199	–1.40686
PN2	ES and CC%AP	0.035029	0.36594
	ES and N%AP	–0.159103	–0.80578
	ES and P%AP	0.00073	0.00372
PN3	ES and CC%AP	–0.138621	–0.37033
	ES and N%AP	0.725476	1.82574

Discussion

The basic principle of the comet assay is that fragmented DNA (apoptosis or necrosis) will create a tail of DNA that trails behind the nucleus after single-cell gel electrophoresis (Ostling and Johansen, 1984; Singh *et al*., 1988). This study was focused on apoptotic DNA fragmentation and the percentage of apoptotic cells (sperm and cumulus) was calculated by simply counting the apoptotic comets, which are characterized by tear-shaped tails. Necrotic cells were ignored.

Recent studies have further elucidated the relationship of cumulus cells, in the context of gene expression for PTGS2 (cyclo-oxygenase 2), HAS2 (hyaluronic acid synthase 2) and GREM1 (gremlin), with the oocyte quality, fertilization rates and embryo quality (McKenzie *et al*., 2004). Raman found a positive relationship between oocyte competence and cumulus cell DNA status (Raman *et al*., 2001). Oocytes that became fertilized after ICSI were positively associated with cumulus cells with increased DNA fragmentation, i.e. greater maturity, and therefore Raman believes that increased apoptosis is related to increased maturity and therefore better fertilization. The study, however, did not include whether or not unfertilized oocytes after ICSI were positively related to even higher levels of apoptosis, and were therefore post-mature. Host *et al*. (2000) are of the opposite opinion, that a lower incidence of apoptosis in individual follicles is associated with better outcome for the oocytes. They believe that apoptosis inhibits maturation, and therefore increased apoptosis is related to decreased maturity and subsequently decreased fertilization (Host *et al*., 2000). The results of the present study, however, did not support Ramans' theory (Raman *et al.,* 2001).They showed that the level of apoptosis in cumulus cells of the oocytes was not comparable between oocytes of different fertilization states (i.e. no fertilization, normal fertilization and abnormal fertilization), and that there was no correlation between the apoptotic level of cumulus cells and the subsequent fertilization of the corresponding oocyte. Both studies agree, though, that patient age had no influence on the degree of apoptosis.

Significant differences in embryo score were apparent between the different fertilized groups, but there was no correlation between the embryo score and apoptosis levels. The embryo score was significantly different between the PN0 and PN2 groups ($P \leq 0.01$). This of course was expected, due to the fact that PN0 oocytes do not divide into an embryo since they are not fertilized. The embryo score comparison was carried out regardless of this to act as a control.

Regarding spermatozoa, there have been different published results. Some groups have found differences in DNA damage levels between infertile and normal males, while others have not (Morris *et al*., 2002). These different results could be due to different methodology used, arising from difficulties in releasing the DNA due to the extremely tight DNA compaction in the sperm head (Morris *et al*., 2002; Sassone, 2002). Moreover, the established relationship of sperm cellular maturity, DNA integrity, chromatin maturity and chromosomal aneuploidy frequency with sperm function including fertilizing potential gave rise to the development of ICSI selection methods that eliminate spermatozoa with DNA defects (Cayli *et al*., 2003).

It has been stated that the use of the ICSI procedure bypasses the need to consider the status of the sperm DNA, requiring only to focus on the oocyte (Raman *et al*., 2001). The authors disagree with this statement. It is of extreme importance to consider all the factors involved, and sperm DNA is a very important and interesting one to be investigated. If a relationship does exist between sperm DNA integrity and the outcome of ICSI it could have major implications in the future of ICSI. Host *et al*. found that apoptotic activity in spermatozoa did affect the degree of fragmentation in embryos, and therefore that male factor does have an impact on the developing embryo (Host *et al*., 2000). Donelly *et al*. also agree stating that 'higher amounts of [sperm DNA] damage are likely to result in poor rates of embryo development and early pregnancy loss' (Donelly *et al*., 2001). Nasr-Esfahani *et al*. (2005) reported that sperm DNA fragmentation does not preclude fertilization. Nonetheless, embryos derived from spermatozoa with DNA damage have a lower potential to reach blastocyst stage.

In the study of Findikli *et al*. (2003), it was suggested that most developmentally blocked or poor quality embryos have intrinsic problems that can probably be traced back to gamete cell environment. If mature oocytes could be chosen on the basis of their cumulus cell DNA status, and the 'best' spermatozoa selected according to their DNA integrity, then ICSI would be entering a new range of possibilities. It is therefore vital to investigate both cumulus cell and sperm DNA status.

As with the cumulus cells, the results showed similar outcomes with the semen. No significant differences were found between the apoptosis levels in native and processed semen, in any of the PN groups, and there was no correlation between processed semen apoptosis levels and fertilization. ICSI was carried out using processed semen, but the assay was also performed on native samples, as a control for comparison. The selection criteria included both primary and secondary infertility, hence the data may be influenced by different parameters.

Conclusively, apoptosis was not found to affect the outcome of ICSI. The idea therefore that oocytes could be selected for ICSI based on the cumulus cell DNA status increasing the chances of a positive outcome needs more investigation, as does the selection of spermatozoa based on its DNA integrity. If it is decided in the future that this is possible and useful, then a method will have to be applied that can assess the cells with no damage and then select the best quality oocytes or spermatozoa for the ICSI to be performed. Sperm preparation should be carried out in such a way as to minimize damage to the spermatozoa. In order to do so, four factors should be considered. Dilution of the samples should be carried out slowly especially with cryopreserved spermatozoa. Any changes in temperature should be gradual, and preparation performed at 37°C. If centrifugation is used, it should be minimal and carried out at the lowest force possible, and finally exposure to potentially toxic material should be considered. When the swim-up technique is used these points can be applied in the following manner. Plastic, glassware and media should be checked for toxicity to spermatozoa, which should not come into contact with rubber as this may immobilize it. Also, media should be maintained at a temperature of 37°C.

Another way to increase the chances of the best quality spermatozoa is during sperm collection. In the case of men who are unable to collect semen by masturbation, non-toxic

condoms should be used, and in other cases a second sample could be collected a few hours after the first (Trounson and Gardner, 2000).

As for oocytes, ovarian stimulation with gonadotrophins is crucial to increasing the yield of high quality oocytes with and increased chance of fertilization. The timing of oocyte collection is important as to maximize the number of mature oocytes collected. The handling of oocytes once collected is also very important. Temperature should be maintained at 37°C, pH changes should be avoided as much as possible and all handling must be aseptic (Trounson and Gardner, 2000).

Acknowledgements

We would like to thank Dr Mohamed Hossein Nasr-Esfahani in Tehran for sharing his sperm lysis buffer with us.

References

Al-Hasani S, Kuepker W, Baschat AA *et al*. 1995 Mini swim-up: a new technique of sperm preparation for intracytoplasmic sperm injection. *Journal of Assisted Reproduction and Genetics* **12**, 428–433.

Bianche PG, Manicardi GC, Urner F *et al*. 1996 Chromatin packaging and morphology in ejaculated human spermatozoa: evidence of hidden anomalies in normal spermatozoa. *Molecular Human Reproduction* **2**, 139–144.

Cayli S, Jakab A, Ovari L *et al*. 2003 Biochemical markers of sperm function: male fertility and sperm selection for ICSI. *Reproductive BioMedicine Online* **7**, 462–468.

Donelly ET, Steele EK, Muclure N, Lewis EM 2001 Assessment of DNA integrity and morphology of ejaculated spermatozoa from fertile and infertile men before and after cryopreservation. *Human Reproduction* **16**, 1191–1199.

Duty SM, Singh NP, Ryan L *et al*. 2002 Reliability of the comet assay in cryopreserved human sperm. *Human Reproduction* **17**, 1274–1280.

Findikli A, Kahraman S, Kumtepe Y *et al*. 2003 Assessment of DNA fragmentation and aneuploidy on poor quality human embryos. *Reproductive BioMedicine Online* **8**, 196–206.

Host E, Mikkelsen AL, Lindenberg S, Smidt-Jensen S 2000 Apoptosis in human cumulus cells in relation to maturation stage and cleavage of the corresponding oocyte. *Acta Obstetrica et Gynecologica Scandinavica* **79**, 936–940.

McKenzie L, Pangas S, Carson S *et al*. 2004 Human cumulus granulosa cell gene expression: a predictor of fertilization and embryo selection in women undergoing IVF. *Human Reproduction* **19**, 2869–2874.

Morris ID, Ilott S, Dixon L, Brison DR 2002 The spectrum of DNA damage in human sperm assessed by single cell gel electrophoresis (comet assay) and its relationship to fertilization and embryo development. *Human Reproduction* **17**, 990–998.

Moustafa MH, Saleh RA, Oder M *et al*. 2002 High levels of apoptosis in ejaculated spermatozoa from infertile men. *Fertility and Sterility* **78** (Suppl. 1), S106.

Nasr-Esfahani M, Salehi M, Razavi S *et al*. 2005 Effect of sperm DNA damage and sperm protamine deficiency on fertilization and embryo development post-ICSI. *Reproductive BioMedicine Online* **11**, 198–205.

Olive PL, Banath JP 1995 Sizing highly fragmented DNA in individual apoptotic cells using the comet assay and a DNA crosslinking agent. *Experimental Cell Research* **221**, 19–26.

Ostling O, Johanson KJ 1984 Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. *Biochemical and Biophysical Research Communications* **123**, 291–298.

Raman RS, Chan PJ, Corselli JU *et al*. 2001 Comet assay of cumulus cell DNA status and the relationship to oocyte fertilization via intracytoplasmic sperm injection. *Human Reproduction* **16**, 831–835.

Ricci G, Perticarari S, Fragonas E *et al*. 2002 Apoptosis in human sperm: its correlation with semen quality and the presence of leukocytes. *Human Reproduction* **17**, 2665–2672.

Sakkas D, Seli E, Bizzaro D *et al*. 2003 Abnormal spermatozoa in the ejaculate: abortive apoptosis and faulty nuclear remodeling during spermatogenesis. *Reproductive BioMedicine Online* **7**, 428–432.

Saleh RA, Agarwal A, Nada EA *et al*. 2002 Negative effects of sperm nuclear DNA damage on the fertility potential of couples with idiopathic and male-factor infertility. *Fertility and Sterility* **78**, Abstracts 0–160, S61.

Sassone-Corsi P 2002 Unique chromatin remodeling and transcriptional regulation in spermatogenesis. *Science* **296**, 2176–2178.

Shen H-M, Dai J, Chia S-E *et al*. 2002 Detection of apoptotic alterations in sperm in subfertile patients and their correlations with sperm quality. *Human Reproduction* **17**, 1266–1273.

Singh NP, McCoy MT, Tice RR, Schneider EL 1988 A simple technique for quantitation of low levels of DNA damage in individual cells. *Experimental Cell Research* **175**, 184–191.

Tomlinson MJ, Mofatt O, Manicardi GC *et al*. 2001 interrelationships between seminal parameters and sperm nuclear DNA damage before and after density gradient centrifugation: implications for assisted conception. *Human Reproduction* **16**, 2160–2165.

Tomsu M, Sharma V, Miller D 2002 Embryo quality and IVF treatment outcomes may correlate with different sperm comet assay parameters. *Human Reproduction* **17** 1856–1862.

Trounson AO, Gardner DK 2000 *Handbook of In Vitro Fertilization*, 2nd edn. CRC Press. Boca Raton, London, New York, Washington DC.

Veeck L 1999 *An Atlas of Human Gametes and Conceptuses*. Parthenon Publishing Group, New York, London.

Received 13 September 2005; refereed 27 September 2005; accepted 11 January 2006.