Full-term pregnancies achieved with ICSI despite high levels of sperm chromatin damage

L.Gandini[1,5], F.Lombardo[1], D.Paoli[1], F.Caruso[1], P.Eleuteri[2], G.Leter[2], R.Ciriminna[3], F.Culasso[4], F.Dondero[1], A.Lenzi[1] and M.Spanò[2]

[1]Laboratory of Seminology and Reproductive Immunology, Department of Medical Pathophysiology and [4]Chair of Health Statistics, Department of Experimental Medicine, University of Rome 'La Sapienza', [2]Section of Toxicology and Biomedical Sciences, ENEA CR Casaccia and [3]ART Unit, CID, Rome, Italy

[5]To whom correspondence should be addressed. E-mail: loredana.gandini@uniroma1.it

BACKGROUND: Sperm DNA integrity is essential for the accurate transmission of genetic information. The clinical significance of this assessment lies in its association with not only natural conception rates, but also the success of assisted reproduction technology (ART). It has been reported that sperm chromatin structure assay (SCSA) identified thresholds for negative pregnancy outcome after ART when the DNA fragmentation index (DFI), previously known as COMPαt, was >30%. METHODS: In a prospective clinical study, we examined 34 male infertile patients, the husbands of women undergoing conventional IVF or ICSI. SCSA and ART were carried out on semen aliquots taken from the same ejaculate. Fertilization rate, embryo quality and pregnancy rates were correlated to SCSA parameters, DFI and highly DNA stainable (HDS) cells. RESULTS: No differences were seen in SCSA parameter values between patients initiating pregnancies and not doing so in either ICSI or conventional IVF. Pregnancies and normal delivery were obtained even with high levels of DFI. CONCLUSIONS: There is still controversy over whether analytical techniques currently in use are able to identify the level of damage to spermatozoa. Large-scale studies should be conducted in different clinical settings to determine the effects of sperm DNA damage on the outcome of ART.

Key words: chromatin damage/ICSI/IVF/sperm chromatin/structure assay

Introduction

Over the last decade, we have seen the increasing use of assisted reproductive technology (ART) to overcome the problem of couple infertility (Katz *et al.*, 2002). ARTs are now routinely proposed in the cases of couples with a severely subfertile male partner who wish to have a child. However, the pregnancy rate is still relatively low and somewhat unpredictable, given the number of variables possibly involved in the multistep process of fertilization, embryo cleavage, implantation and pregnancy.

The methods of gamete manipulation used in ART are rapidly proliferating, and in some instances outpacing the underlying science (Schultz and Williams, 2002). The original indications of ICSI, initially suggested for cases of severe male factor infertility, have been expanded. There are controversies about the safety of the procedure, which bypasses all processes of natural selection from sperm–oocyte physical interaction, still present in conventional IVF, as it relies on the direct injection of a sperm into the oocyte cytoplasm. There are potential concerns relating to obstetric outcome, chromosomal abnormalities, congenital malformations and developmental abnormalities in ICSI-born progeny. Following recent strong

warnings about the potential risks of ART (especially ICSI) for a number of reasons (Hansen *et al.*, 2002; Schieve *et al.*, 2002; Stromberg *et al.*, 2002; DeBaun *et al.*, 2003; Moll *et al.*, 2003; Orstavik *et al.*, 2003), scientific debate has become quite passionate (Bonduelle *et al.*, 2003; Retzloff and Hornstein, 2003; Tournaye, 2003). ICSI has made the conventional criteria of concentration, motility and morphology for assessment of sperm quality irrelevant. The need for new assays for evaluation of sperm health has thus become a priority.

The identification of sound, suitable semen and sperm characteristics enabling prediction of the chances of successful outcome after ART is an area of active research. Intuitively, the more intact and functional the spermatozoon, the higher the chances of delivering a healthy progeny. Standard semen analysis is therefore a mandatory test in the investigation of the infertile couple; however, its predictive value in ART is quite limited (Host *et al.*, 2001). Moreover, this analysis gives no insight into the integrity of the genome of the male gamete. In addition to chromosome aneuplodies, more subtle abnormalities at the DNA and/or chromatin level can adversely affect normal and assisted fertilization, as well as the normal development of the embryo, fetus and child (Brinkworth,

2000). The presence of sperm with DNA fragmentation and chromatin abnormalities in human ejaculates is well documented (Agarwal and Said, 2003; Sakkas *et al.*, 2003), in particular in men with poor semen quality (Lopes *et al.*, 1998; Gandini *et al.*, 2000; Irvine *et al.*, 2000; Hammadeh *et al.*, 2001b; Sakkas *et al.*, 2002; Saleh *et al.*, 2002; Erenpreisa *et al.*, 2003; Muratori *et al.*, 2003). It should be highlighted that, in some cases of severe male factor infertility, a significant proportion of spermatozoa injected into oocytes may therefore contain fragmented DNA. Injection of oocytes with spermatozoa with abnormal chromatin will probably result in failure of sperm decondensation and fertilization. In addition, due to the negative association between semen analysis parameters and sperm with DNA damage, and as extremely poor semen samples are the indication for ART, there is a high probability that these altered sperm may be used for oocyte injection, resulting in poor fertilization and/or cleavage rates. There is therefore a real risk of accidentally selecting genetically abnormal sperm which may appear motile and morphologically normal.

It has been demonstrated that chromatin-damaged human spermatozoa are able to form normal pronuclei in oocytes after ICSI just like sperm with an intact genome (Twigg *et al.*, 1998). As the consequences of this for embryo development are not entirely known, a general consensus is emerging that DNA/chromatin defective sperm assessment should be integrated as a complementary diagnostic tool in any ART programme (Perreault *et al.*, 2003).

Sperm DNA integrity can be determined quickly and accurately using a range of techniques (reviewed in Evenson *et al.*, 2002; Agarwal and Said, 2003; Perreault *et al.*, 2003; Sakkas *et al.*, 2003) which also seem to have strong prognostic power in predicting successful ART outcomes. This is demonstrated by the accumulating body of evidence suggesting that disturbances in the organization of genomic material in sperm nuclei, evaluated using a variety of assays, are negatively correlated with spermatozoa fertility potential, whether *in vivo* (Evenson *et al.*, 1999; Hacker-Klom *et al.*, 1999; Spanò *et al.*, 2000; Bonde *et al.*, 2003; Carrell *et al.*, 2003) or *in vitro* (Hoshi *et al.*, 1996; Sun *et al.*, 1997; Hammadeh *et al.*, 1998; Lopes *et al.*, 1998; Sakkas *et al.*, 1998; Filatov *et al.*, 1999; Esterhuizen *et al.*, 2000; Host *et al.*, 2000; Larson *et al.*, 2000; Evenson *et al.*, 2002; Morris *et al.*, 2002; Tomsu *et al.*, 2002; Virant-Klun *et al.*, 2002; Benchaib *et al.*, 2003; Larson-Cook *et al.*, 2003). However, even though the trend between the percentage of sperm with abnormal chromatin/DNA damage and ART outcome is quite clear, discrepancies still emerge when the fraction of defective sperm is associated with fertilization rate, embryo quality, pregnancy rate, and whether the technique has been used in the context of IVF or ICSI. When the flow cytometric (FCM) sperm chromatin structure assay (SCSA) was applied, thresholds (calculated on neat semen) were found for negative pregnancy outcome after ART which had not been identified using conventional semen parameters (Larson *et al.*, 2000; Saleh *et al.*, 2003; Larson-Cook *et al.*, 2003).

The objective of this study was to determine possible relationships between SCSA parameters evaluated on both neat semen and the processed aliquot to be used in the ART programme, collected on the day of the oocyte pick-up, and fertilization rate, embryo quality and pregnancy rate following ICSI and IVF.

Materials and methods

Patients

We examined 34 infertile male patients, the partners of women who were undergoing IVF or ICSI. The patients had suffered from primary infertility for at least 3 years, were aged 29–45, and had not been treated medically or surgically in the 3 months prior to the study.

Patients were divided into two groups according to the ART technique employed. Group A, with 12 patients, underwent IVF; and group B, consisting of 24 patients, underwent ICSI.

Group A female partners underwent IVF either because of a bilateral Fallopian tube obstruction, or following at least four failed attempts at intrauterine insemination with ovarian stimulation under ultrasonographic and hormonal control.

Group B female partners were normal, but seminal fluid of the male partners was characterized by moderate to severe oligoasthenoteratozoospermia (OAT) and so they underwent ICSI. Two male partners had a sperm concentration/ml of 80 and 110×10^6 ml, respectively. In these cases, ICSI was performed due to two previous failed attempts at IVF (fertilization rate <30%).

The median age of female partners was 34.8 years (range 26–44) in group A and 35.1 years (range 30–44) in group B.

All patients had a normal peripheral blood karyotype, and patients with severe OAT were free of Yq microdeletion.

All patients gave their informed consent prior to the study, which was approved by the Institutional Review Board of our University.

Semen examination and in vitro treatment

On the day of oocyte pick-up, semen samples were collected by masturbation into sterile plastic jars after 3–5 days of sexual abstinence. They were allowed to liquefy for 30 min at room temperature (22°C) and were then evaluated according to the WHO criteria (World Health Organization, 1999). The variables taken into consideration were: ejaculate volume (ml), sperm concentration ($n \times 10^6$/ml), forward motility (%) and morphology (% atypical forms). After microscopic evaluation, the samples were prepared for the assisted reproduction procedure, using swim-up for IVF and PureSperm (BioCare, Europe) gradients for ICSI.

Samples for IVF were washed twice as follows: the semen sample was diluted 1:2 with Earle's salt solution (ESS; Sigma Chemical Co, St Louis, MO) supplemented with 5% human serum albumin (HSA) (BioCare, Europe) and centrifuged for 10 min at 300 *g*; the supernatant was then discarded and an aliquot of 0.5 ml of ESS was layered on the pellet; after incubation for 30 min at 37°C in 5% CO_2, the supernatant was recovered.

Samples for ICSI were prepared as follows: PureSperm 100% was diluted with ESS-HSA to obtain 40 and 80% dilutions. Several PureSperm gradient columns were prepared in 5 ml Falcon tubes by gently layering 1 ml of each solution, starting from the 100% fraction at the bottom; 0.5 ml of the semen sample was stratified on top of the discontinuous PureSperm gradient columns and centrifuged for 25 min at 300 *g*. After centrifugation, the 100% PureSperm fraction was recovered and after thorough washing was used for insemination.

One aliquot of each of the raw semen and processed spermatozoa obtained by swim-up or PureSperm gradient were treated for evaluation of sperm chromatin structure. Briefly, after washing in TNE buffer (0.15 mol/l NaCl, 0.01 mol/l Tris–HCl, 1 mmol/l EDTA

pH 7.4), pellets were re-suspended in TNE buffer containing 10% glycerol at a final sperm concentration of 2×10^6/ml and transferred to Eppendorf snap-cap tubes. The tubes were kept on dry ice until transfer to an ultra-cold freezer (–80°C) for storage until FCM analysis.

Assisted reproduction procedures

Ovarian stimulation was induced using recombinant FSH therapy at a dosage personalized for each patient for the first 5 days. Further FSH doses and the timing of the administration of 10 000 IU of HCG were adjusted according to the usual criteria of follicular maturation determined by ultrasound and estradiol findings. Pre-treatment with s.c. buserelin (2×100 µg, Suprefact, Hoechst Marion Roussel) was started in the midluteal phase of the cycle prior to stimulation.

Oocytes were retrieved 36 h after HCG administration by transvaginal ultrasound-guided aspiration.

Embryo quality was assessed 48 h after fertilization using the following grading system: grade 1, even, regular spherical blastomeres, intact zona, very few fragments (<10%); grade 2, uneven or irregular sized blastomeres, intact zona, no more than 20% fragments; grade 3, fragmentation of no more than 50% of blastomeres, intact zona; and grade 4, >50% fragmentation, uneven or irregular shaped blastomeres (Veeck, 1991; Desai et al., 2000).

All embryo transfers were performed 2 days after oocyte retrieval using Fryedman catheters (CCD Laboratories, Paris, France). The luteal phase was supported with progesterone (Crinone® 8%; ARES Serono SA, Rome) administered daily by the vaginal route, starting on the evening of embryo transfer.

Serum βHCG levels were measured 12 days after embryo transfer. Clinical pregnancy was confirmed by transvaginal ultrasound 5 weeks after embryo transfer.

FCM SCSA

SCSA was applied following the procedure described in literature (Spanò et al., 2000; Evenson et al., 2002). On the day of analysis, samples were quickly thawed in a 37°C water bath and used immediately. A total of $1–2 \times 10^6$ cells were treated with a pH 1.2 detergent solution containing 0.1% Triton X-100, 0.15 mol/l NaCl and 0.08 mol/l HCl for 30 s and then stained with 6 mg/l of purified acridine orange (AO; Molecular Probes, Eugene, OR) in a phosphate–citrate buffer, pH 6.0. Cells were analysed by a FACScan flow cytometer (Becton Dickinson, San Josè, CA), equipped with an air-cooled argon ion laser. A total of 5000 events were accumulated for each measurement. Under these experimental conditions, AO intercalated with double-stranded DNA emits green fluorescence and AO associated with single-stranded DNA emits red fluorescence when excited with a 488 nm light source. Sperm chromatin damage can thus be quantified by FCM measurements of the metachromatic shift from green (native, double-stranded DNA) to red (denatured, single-stranded DNA) fluorescence and displayed as red (fragmented DNA) versus green (DNA stainability) fluorescence intensity cytogram patterns.

Adopting the guidelines described in a recent publication (Evenson et al., 2002) and using the dedicated SCSASoft software (SCSA Diagnostics, Brookings, SD) for off-line data analysis, we expressed the extent of DNA denaturation in terms of the DNA fragmentation index (DFI), the ratio of red to total (red plus green) fluorescence intensity which previously was known as COMPαt. This is the level of denatured DNA over total DNA. The DFI value was calculated for each sperm cell in a sample, and the percentage of abnormal sperm with detectable DFI values was evaluated from the resulting DFI frequency distribution histogram.

Table I. Mean (±SD) and significance of sperm and SCSA parameters of the 12 IVF patients

	Raw semen	After swim-up	P
Concentration/ml ($n \times 10^6$)	87.1 ± 28.3	15.4 ± 9.8	<0.001
Forward motility (%)	47.1 ± 7.8	88.3 ± 5.4	<0.001
Atypical forms (%)	57.5 ± 3.1	31.9 ± 4.9	<0.001
DFI (%)	12.0 ± 4.0	5.5 ± 4.3	<0.001
HDS (%)	8.8 ± 4.2	3.3 ± 2.5	<0.001

We also considered the fraction of highly DNA stainable (HDS) cells. Their percentage was calculated by setting an appropriate gate on the scattergram (abscissa: red fluorescence, fragmented DNA; ordinate: green fluorescence, native DNA stainability) and considering as immature spermatozoa those events exhibiting a green fluorescence intensity higher than the upper border of the main cluster, which represents the sperm population with non-detectable DFI. For flow cytometer set-up and calibration, aliquots were used from a normal human ejaculate sample retrieved from our laboratory repository. Samples were measured twice, blind, and the code was broken only at the end of the measurement series. The results reported refer to the mean value of the two FCM measurements.

Statistical analysis

Descriptive statistical analysis was performed separately on the two groups. Comparisons of mean values were calculated from the appropriate test (paired t-test and Wilcoxon test, t-test for independent samples and Mann–Whitney test) for the variables HDS, DFI, motility and atypical forms. A logistic regression was performed separately for the two groups, to assess the association of pregnancy and DFI and embryo grading. Finally, correlation (Pearson's r and Spearman correlation) of the variables with fertilization rate and DFI was calculated. Statistical analyses were also performed after logarithmic transformation of percentage variables.

Results

We studied 34 couples, of whom 12 underwent IVF-ET (group A) and 22 ICSI (group B).

Group A (IVF-ET)

Table I reports the mean ± SD of sperm and SCSA parameters of the samples before and after the swim-up procedure. The improvement in sperm motility and atypical forms was statistically significant ($P < 0.001$). Differences were also statistically significant ($P < 0.001$) for the SCSA parameters DFI and HDS. An improvement in sperm chromatin quality [reduction of percentage sperm with damaged DNA (DFI fraction) and percentage spermatozoa with high DNA stainability (HDS fraction)] was seen for all samples after swim-up (Table II).

The mean ± SD of oocytes inseminated and fertilized were 10.0 ± 4.8 and 7.5 ± 3.9, respectively; the fertilization rate was 68.8%, and three pregnancies (25%) were obtained in this group. All three reached full term and five healthy children were born (two single births and one set of triplets). Table II shows, for each patient, results of DFI and HDS in raw semen and after swim-up, fertilization rate and embryo grading

Table II. DFI and HDS percentage before and after swim-up, number of oocytes inseminated and fertilized, fertilization rate and full-term pregnancy outcome for each IVF patient

Patient	DFI (%)		HDS (%)		Oocytes inseminated (*n*)	Oocytes fertilized (*n*)	Fertilization rate (%)	Pregnancy
	Raw	Swim-up	Raw	Swim-up				
1	16.5	16.1	7.8	2.8	7	5	71.4	No
2	7.9	2.1	3.3	0.6	10	8	80.0	No
3	16.1	5.3	13.0	5.7	15	12	80.0	Yes
4	9.3	8.2	7.2	8.6	16	12	75.0	No
5	9.3	3.1	6.6	5.2	11	9	81.8	No
6	11.3	9.5	13.4	4.3	4	3	75.0	Yes
7	5.9	2.7	6.3	0.8	16	13	81.3	Yes
8	16.3	1.4	5.2	0.6	14	9	64.3	No
9	18.8	2.0	4.0	0.7	1	0	0.0	No
10	15.0	3.7	9.5	1.5	6	4	66.7	No
11	11.3	8.5	12.7	4.9	11	8	72.7	No
12	13.1	3.7	16.5	3.6	9	7	77.8	No

Table III. Mean (±SD) and significance of sperm and SCSA parameters of the 22 ICSI patients

	Raw semen	After PureSperm	*P*
Concentration/ml ($\times 10^6$)	24.4 ± 26.3	12.8 ± 15.0	<0.01
Forward motility (%)	22.5 ± 14.3	51.6 ± 31.0	<0.001
Atypical forms (%)	74.4 ± 12.9	58.0 ± 19.9	<0.001
DFI	28.1 ± 14.5	24.2 ± 19.9	NS
HDS	17.8 ± 11.1	9.2 ± 7.5	<0.001

grouped for obtained and failed pregnancies. There was no significant correlation between DFI/HDS results before and after swim-up and the fertilization rate ($P > 0.05$). No significant differences were seen between DFI and HDS fractions of patients achieving and not achieving pregnancy.

Group B (ICSI)

The mean ± SD of sperm and SCSA parameters of the samples before and after PureSperm selection are reported in Table III. The improvement in sperm motility and atypical forms was statistically significant ($P < 0.001$). In this group, the difference in DFI values between raw semen and after Pure Sperm gradient was not statistically significant. However, the difference was statistically significant for HDS values ($P < 0.001$).

The mean ± SD of inseminated and fertilized oocytes were 9.0 ± 5.2 and 6.2 ± 4.1, respectively. The fertilization rate was 70.0%. In this group, nine pregnancies were achieved (40.9%). All reached full term and 13 healthy children were born (seven single births and two sets of triplets). Five of these pregnancies were initiated by patients whose DFI value was >27%. No statistically significant differences were seen for DFI and HDS values when the group achieving pregnancy was compared with the group who failed to father a child (Table IV). No correlation was found between percentage of spermatozoa with altered DFI and HDS and the fertilization rate and embryo grading in patients achieving and not achieving a pregnancy by ICSI (Table IV). It is worth highlighting the heterogeneity of the SCSA values on the neat samples of the nine male partners

of couples achieving pregnancy after ICSI, with DFI ranging from 13.5 to 66.3%.

No significant differences in the age of male and female partners of the couples and number of oocytes retrieved were seen in the two groups (data not shown).

Discussion

From the literature available, no difference has been reported between levels of embryo loss after ICSI and IVF. For example, the European IVF monitoring programme (EIM) for the European Society of Human Reproduction and Embryology (ESHRE) recently reported that, for treatments initiated during 1997, the clinical pregnancy rate per transfer for IVF was 26.1% and the delivery rate per embryo transfer 20.9%, whereas for ICSI, the corresponding rates were 26.4 and 21.5% (ESHRE, 2001). Our results are in general agreement with these figures. However, Sanchez *et al.* (1996) reported a miscarriage rate following ICSI greater than expected in conventional IVF, possibly reflecting the use of genomically compromised spermatozoa which conferred irreparable DNA damage to the embryo, resulting in its abortion. Moreover, a higher frequency of numerical chromosome abnormalities after ICSI than with IVF has been demonstrated (Macas *et al.*, 1996) and there is evidence that genetic damage in paternal gametes could predispose offspring to disease that does not manifest itself until later in life, particularly childhood cancer (Sorahan *et al.*, 1997).

As sperm genetic integrity has been implicated in fertilization and embryo development failures (Ahmadi and Ng, 1999; Cho *et al.*, 2003), several groups have also analysed sperm nuclear integrity in terms of DNA/chromatin damage and its relationship to male infertility and ART outcome, why nuclear damage originates in certain males and how it influences the mature spermatozoon. Several methods are now available to detect sperm chromatin abnormalities, and their value and robustness are under active scrutiny (Irvine *et al.*, 2000; Evenson *et al.*, 2002; Erenpreisa *et al.*, 2003; Perreault *et al.*, 2003).

Table IV. DFI and HDS percentage, fertilization rate and embryo grading for each ICSI patient

Patients	DFI before PureSperm (%)	DFI after PureSperm (%)	HDS before PureSperm (%)	HDS after PureSperm (%)	Fertilization rate (%)	Embryo grading 1	Embryo grading 2
Full-term pregnancy							
2	13.9	11.5	6.5	4.6	50.0	3	1
3	33.2	60.4	9.6	7.8	61.5	4	0
5	26.0	25.4	14.1	10.6	75.0	2	2
9	37.7	8.6	11.9	10.5	81.8	2	1
12	56.0	33.4	8.3	4.9	83.3	3	0
14	20.7	1.7	12.6	2.2	76.5	3	0
18	39.1	11.7	29.5	3.4	66.7	0	3
19	66.3	55.2	34.5	22.3	52.9	4	0
21	13.5	5.4	13.6	5.2	41.7	2	2
Mean	34.0	23.7	15.6	7.9	65.5	2.6	1.0
SD	18.1	21.7	9.7	6.1	14.9	1.2	1.1
Failure							
1	20.8	47.1	6.3	7.7	100	1	1
4	41.0	7.5	16.6	3.9	0	0	0
6	8.8	7.7	38.6	25.9	81.3	0	0
7	36.8	30.6	12.6	8.2	100	1	0
8	21.7	16.3	15.1	5.3	75.0	1	2
10	9.2	13.0	5.9	6.7	70.0	3	0
11	17.4	24.1	23.8	25.1	62.5	0	2
13	27.9	3.7	15.0	2.4	100	3	0
15	52.9	39.1	32.6	11.6	92.3	3	0
16	8.0	4.0	3.7	1.0	100	2	0
17	20.1	27.0	24.0	17.2	20	2	0
20	35.6	71.4	41.1	1.5	50	1	2
22	26.6	28.1	15.9	15.0	100	0	4
Mean	25.1	24.6	19.3	10.1	73.2	1.3	0.8
SD	13.6	19.6	12.1	8.4	32.8	1.2	1.3

Not only sperm genetic integrity (Ahmadi and Ng, 1999) but also protamine deficiency can have a negative impact on male fertility, resulting in the failure of fertilization and embryo development. Knock-out mice for protamine 2 expression showed an increased rate of sperm with DNA breaks (as seen by the COMET assay) and embryo failure after ICSI (Cho *et al.*, 2003), confirming the pivotal role protamines play in safeguarding DNA integrity.

The impact of sperm DNA/chromatin integrity on ART outcome has been studied using a variety of techniques. Using aniline blue staining, a method allowing detection of chromatin abnormalities in terms of excessive residual histones in the sperm chromatin assembly, it has been reported that chromatin condensation is a good predictor for IVF outcome, as fertilization and pregnancy rate were higher when the fraction of sperm with abnormal chromatin was <20% (Hammadeh *et al.*, 1998). However, the same group found no significant relationships for fertilization, cleavage, implantation and pregnancy rates when applying the aniline blue technique to ICSI outcome (Hammadeh *et al.*, 1999).

Using the AO method (Tejada *et al.*, 1984), Hoshi *et al.* (1996) reported that when ≥50% of spermatozoa exhibited green AO fluorescence, IVF was always successful, whereas if AO green fluorescent sperm were <50%, no full-term pregnancies resulted, even after successful ICSI fertilization. Virant-Klun *et al.* (2002) noted that when the level of red fluorescent spermatozoa increased, there was a significantly lower fertilization rate after ICSI and a significantly higher number of embryos were arrested. However, no correlation was found between abnormal spermatozoa pregnancy rate and live birth rate achieved by ICSI, except in patients having no spermatozoa with single-stranded DNA, in whom the pregnancy rate was significantly higher. Katayose *et al.* (2003) used a modified AO procedure involving the derivative diamide-AO, which oxidizes nuclear protamine thiol groups. They noted a positive correlation between the fertilization rate after conventional IVF and the fraction of normal green fluorescent sperm. However, Angelopoulos *et al.* (1998) found that AO staining did not predict fertilization efficiency or pregnancy outcome in IVF cycles, and no correlations were found between chromatin decondensation and fertilization rate after ICSI (Hammadeh *et al.*, 2001a).

The chromomycin A3 (CMA3) method can detect sperm chromatin abnormalities, always at the level of histone-to-protamine substitution but by examining the relative protamine abundance: decondensed, protamine-depleted spermatozoa will appear fluorescent under the microscope. Sakkas *et al.* (1996), taking normal semen values as normal morphology >20%, CMA3 fluorescence <30% and endogenous nicks <10%, reported that in a series of ICSI patients, abnormal semen samples produced more than double the number of unfertilized oocytes containing condensed spermatozoa than did normal semen samples, but no relationships were found with the fertilization rate. Moreover, CMA3 fluorescence (but not aniline blue staining) correlated with post-ICSI fertilization outcome (Razavi *et al.*, 2003)

Sperm DNA damage can be measured directly by terminal deoxynucleotidyltransferase-mediated dUTP nick end label-

ling (TUNEL) and single-cell gel electrophoresis (COMET) assay. Using the TUNEL assay, negative associations were found between the percentage sperm with DNA fragmentation and fertilization and embryo cleavage rates after IVF (Sun *et al.*, 1997). A significant negative association was also found between the percentage sperm with DNA fragmentation and ICSI fertilization rate (Lopes *et al.*, 1998). Benchaib *et al.* (2003) found that a high proportion of sperm with fragmented DNA (>10%) was a negative factor for achievement of pregnancy when ICSI was performed, but there was no relationship when conventional IVF was carried out. However, Host *et al.* (2000) found that the level of DNA strand breaks was predictive of the success rate in IVF but not in ICSI.

Using the COMET assay, Tomsu *et al.* (2002) noted that the COMET head and tail DNA parameters could be considered potentially useful predictors of embryo quality and IVF outcomes, especially in couples with unexplained subfertility. It has also been shown that high loads of DNA damage were predictive of embryo development failure after ICSI (Morris *et al.*, 2002).

SCSA is a quantitative assessment of sperm chromatin integrity defined as susceptibility of DNA to acid-induced denaturation *in situ*. It has been shown that the fraction of abnormal sperm detected by SCSA is higher in men with infertility problems (Zini *et al.*, 2001a,b; Saleh *et al.*, 2002; Schmid *et al.*, 2003).

Two large independent studies (Evenson *et al.*, 1999; Spanò *et al.*, 2000) on the relationship between SCSA results and sperm fertilization capacity have been carried out in the USA and Europe. Both demonstrated that when >30% of sperm have abnormal chromatin as evaluated by SCSA, human male fertility is hampered, independent of sperm number, morphology and motility. SCSA also seems correlated with embryo development failures (Evenson *et al.*, 1999; Bonde *et al.*, 2003). Recently, SCSA has also started to be applied in the context of ART.

In the three SCSA papers published to date (Larson *et al.*, 2000; Larson-Cook *et al.*, 2003; Saleh *et al.*, 2003), no full-term pregnancies (after ICSI, IVF or IUI) were obtained when the DFI fraction was >28%. This leads to the conclusion that SCSA-derived thresholds significantly predict negative pregnancy outcomes in couples attempting pregnancy via ART. In particular, Larson *et al.* (2000) reported a pregnancy rate of 24% per oocyte transferred if COMPαt (now termed DFI) was <27%, and 0% if COMPαt was >27%; Saleh *et al.* (2003) obtained 30% of pregnancies for IVF and 25% for ICSI; Larson-Cook *et al.* (2003) reported 29 pregnancies out of 89 FIVET/ICSI (32.5%); 28 pregnancy were obtained with a DFI <27%; one ICSI patient achieved a biochemical pregnancy with >27% DFI, but subsequently lost it before ultrasound.

The results of our ART programmes and SCSA-assessed sperm integrity do not indicate a clear predictive power for DFI (and HDS) fraction for ICSI fertility rate, embryo quality and pregnancy rate. The mean DFI value for the nine men who fathered a child (pregnancy rate 40.9%) was 32.1% (23.7% after PureSperm separation). This value was no different from

that of the group of men not fathering a child (25.1%; 24.6% after PureSperm).

The categories proposed by Evenson *et al.* (2002) for individual fertility potential according to DFI fraction are: excellent <15%, good 15–24%, fair 25–30%, and poor >30% DFI, and if HDS is >15%, the fertility potential is downgraded at least one category, e.g. from excellent to good. Using these categories, five of the nine ICSI successes were characterized by excellent SCSA quality after PureSperm, and three were above the DFI threshold proposed by other authors (Larson-Cook *et al.*, 2003; Saleh *et al.*, 2003). This is in agreement with Larson-Cook *et al.* (2003) who reported that fertilization rate, cleavage rate and blastulation rate were not significantly related to SCSA parameters. Fertilization rates in our study were similar to those of Larson-Cook *et al.* (2003). However, Saleh *et al.* (2003) found that DFI levels were negatively correlated with fertilization and embryo quality after IVF and ICSI.

The semen quality of the IVF group is notably higher in terms of concentration, morphology, motility and chromatin structure integrity than seen in the ICSI group. In the IVF group, SCSA analysis on raw semen demonstrated seven excellent and five good samples, whereas after swim-up 11 were excellent and one was good. Pregnancies were obtained for DFI values of <10%, especially in the swim-up samples used for fertilization, well under the proposed threshold of 30–40%. However, this alone does not ensure that pregnancy is achieved, as other sperm factors must be involved in the fertilization process.

DFI values of individuals who fathered a child (11.1% raw semen, 5.8% after swim-up) were not statistically different from those who did not (13.1% raw semen, 5.4% after swim-up). We are therefore unable to draw any definitive conclusion on the predictive power of SCSA DFI, as donor semen characteristics were always of a high quality even where IVF pregnancies were obtained with ejaculates in which DFI was below the proposed threshold value for *in vitro* and *in vivo* fertility.

The swim-up technique very effectively separated the fraction of highly motile cells which were characterized, in all instances, by better sperm chromatin integrity. Mean DFI was 12.0% in the neat semen: this figure dropped to 5.5% after swim-up, confirming the evidence of previous experiments (Spanò *et al.*, 1999). The swim-up procedure discriminates a subpopulation of highly motile cells with improved morphology, consistently selecting spermatozoa to give a notable improvement in sperm chromatin features as evaluated by SCSA. In particular, the fraction of DFI and HDS cells markedly decreased in all samples, and the mean value was less than half that observed in neat semen samples.

The swim-up methodology is considered that closest to the natural selection occurring in the female genital tract, emphasizing that motility and migration in a medium different from seminal plasma represent an effective, selective, non-invasive procedure to select sperm with the best chance of achieving fertilization. In contrast, PureSperm gradient, although powerful in its selection of motile and good morphology spermatozoa (Sakkas *et al.*, 2000a; Tomlinson

et al., 2001), was less effective in enriching the cell population with a higher fraction of sperm with normal chromatin structure: in fact, DFI was 28.1% in neat semen and dropped to 24.2% after PureSperm. In our series, some patients showed a higher DFI value after Puresperm than in raw semen. This has also been noted by other groups, indicating that the potential detrimental effect of density gradient centrifugation on sperm DNA integrity may be related to initial semen quality (Zini *et al.*, 2000a,b), which in ICSI patients is generally poor. However, the gradient separation procedure was quite effective in reducing the sample's HDS fraction.

In our opinion, it is not surprising that the invasiveness of the ICSI procedure allows even low quality sperm the chance to initiate a successful pregnancy. Successful pregnancies have been reported in ICSI even using testicular spermatids (Mansour *et al.*, 2003), whose chromatin structure, by definition, is quite different from that acquired by sperm after spermiogenesis and epididymal maturation. Other reports in the literature using different approaches to assess sperm chromatin structure and DNA integrity [e.g. aniline blue staining (Hammadeh *et al.*, 1998, 1999) or TUNEL assay (Host *et al.*, 2000)] have found such characteristics to be more predictive in IVF and much less so in ICSI. Apparently, sperm chromatin integrity becomes particularly relevant when the contact between the two gametes occurs in a more natural way, such as in normal circumstances or in conventional IVF. We can speculate that in these circumstances, selective pressures operate to avoid the development of an embryo derived from sperm with a high load of genetic damage (Morris *et al.*, 2002). This can be different in ICSI, where sperm are forced into the oocyte and the possibility of selecting an unwanted, genetically defective sperm is much higher. As it is likely that sperm with high DNA damage levels have contributed to successful fertilization and *in vitro* development, potential adverse effects remain to be clarified (Sakkas *et al.*, 2000b).

Clearly, the early stages of zygote formation are completely different between ICSI and IVF (Hewitson *et al.*, 2003; Terada *et al.*, 2003) and the consequences of this are not well understood. We know that heavily damaged sperm can fertilize hamster oocytes and form pronuclei at the same rate as an intact sperm (Twigg *et al.*, 1998). ICSI may be facilitating the transfer of genetic disorders to future generations by bypassing all natural hurdles for sperm selection without imposing more pertinent selection criteria. Sperm DNA quality is vital to the future offspring, irrespective of whether the child is conceived naturally, by IVF or by ICSI. Certainly, the biological impact of an abnormal sperm chromatin structure depends on the combined effects of the extent of DNA or chromatin damage in the spermatozoa and the capacity of the oocyte to repair that damage. Therefore, if spermatozoa selected from samples with extensively damaged DNA are used for IVF, the oocyte repair capacity may be inadequate, leading to a low rate of embryonic development and high early pregnancy loss. This concept is quite intuitive; however, although we are now reasonably able to assess the damage level of a sperm population, we cannot assess the repair capability of the oocyte and do not know if there are oocytes capable of repairing even heavily DNA damaged sperm. Neither can the possibility of selection, within a sample characterized by a high DFI fraction, of sperm with limited DNA damage compensated by the oocyte repair capabilities, be excluded.

We are aware that our study size is quite limited as the total number of patients who underwent ART was small. However, we wish to stress that for the first time, ICSI term pregnancies were obtained even with semen samples characterized by a high SCSA DFI (>30%). As fertilization is a probabilistic event between two individual gametes, we expect to find a probabilistic function describing the phenomenon instead of an all-or-nothing threshold effect for unequivocal pregnancy failure prediction. The assays available to measure abnormality or change in sperm chromatin structure or DNA have been linked most commonly with reductions in fertilization potential, but we are only beginning to evaluate their prognostic power in ART programmes. To quote some remarks of the Breakout Group on the integration of new tests for sperm genetic integrity into semen analysis (Perreault *et al.*, 2003): 'Few tests have found acceptance and routine integration into clinical settings or field studies because of technical concerns, the lack of full understanding of the biological mechanisms behind the endpoints, the lack of standardized methodologies and of consensus protocols to challenge their predictive value for abnormal reproductive outcomes of clinical importance'. Before a large-scale conclusion can be reached, randomized studies in the future must determine the effects of sperm DNA damage on the outcome of ART, and of ICSI in particular, in different clinical settings.

Acknowledgements

References

Agarwal A and Said T (2003) Role of sperm chromatin abnormalities and DNA damage in male infertility. Hum Reprod Update 9,331–345.

Ahmadi A and Ng SC (1999) Developmental capacity of damaged spermatozoa. Hum Reprod 14,2279–2285.

Angelopoulos T, Moshel Y.A, Lu L, Macanas E, Grifo JA and Krey LC (1998) Simultaneous assessment of sperm chromatin condensation and morphology before and after separation procedures: effect on the clinical outcome after in vitro fertilization. Fertil Steril 69,740–747.

Benchaib M, Braun V, Lornage J, Hadj S, Salle B, Lejeune H and Guerin JF (2003) Sperm DNA fragmentation decreases the pregnancy rate in an assisted reproductive technique. Hum Reprod 18,1023–1028.

Bonde JP, Hjollund HI, Henriksen TB, Jensen TK, Spanò M, Kolstad H, Giwercman A, Storgaard L, Ernst E and Olsen J (2003) Epidemiologic evidence on biological and environmental male factors in embryonic loss. Adv Exp Med Biol 518,25–35.

Bonduelle M, Ponjaert I, Steirteghem AV, Derde MP, Devroey P and Liebaers I. (2003) Developmental outcome at 2 years of age for children born after ICSI compared with children born after IVF. Hum Reprod 18,342–350.

Brinkworth MH (2000) Paternal transmission of genetic damage: findings in animals and humans. Int J Androl 23,123–135.

Carrell DT, Liu L, Peterson CM, Jones KP, Hatasaka HH, Erickson L and Campbell B (2003) Sperm DNA fragmentation is increased in couples with unexplained recurrent pregnancy loss. Arch Androl 49,49–55.

Cho C, Jung-Ha H, Willis WD, GouldingEH, Stein P, Xu Z, Schultz RM, Hecht NB and Eddy EM (2003) Protamine 2 deficiency leads to sperm DNA damage and embryo death in mice. Biol Reprod 69,211–217.

DeBaun MR, Niemitz EL and Feinberg AP (2003) Association of in vitro fertilization with Beckwith–Wiedemann syndrome and epigenetic alterations of LIT1 and H19. Am J Hum Genet 72,156–160.

Desai NN, Goldstein J, Rowland DY and Goldfarb JM (2000) Morphological evaluation of human embryos and derivation of an embryo quality scoring system for day 3 embryos: a preliminary study. Hum Reprod 15,2190–2196.

Erenpreisa J, Erenpreiss J, FreivaldsT, Slaidina M, Krampe R, Butikova J, Ivanov A and Pjanova D (2003) Toluidine blue test for sperm DNA integrity and elaboration of image cytometry algorithm. Cytometry 52A,19–27.

ESHRE. (2001) The European IVF-monitoring programme (EIM), for the Europaen Society of Human Reproduction and Embryology (ESHRE). Assisted reproductive technology in Europe, 1997. Results generated from European registers by ESHRE. Hum Reprod 16,384–391.

Esterhuizen AD, Franken DR, Lourens JG, Prinsloo E and van Rooyen LH (2000) Sperm chromatin packaging as an indicator of in-vitro fertilization rates. Hum Reprod 15,657–661.

Evenson DP, Jost LK, Marshall D, Zinaman MJ, Clegg, E, Purvis K, De Angelis P and Clausen OP (1999) Utility of the sperm chromatin structure assay (SCSA) as a diagnostic and prognostic tool in the human fertility clinic. Hum Reprod 14,1039–1049.

Evenson DP, Larson KL and Jost LK (2002) Sperm chromatin structure assay: Its clinical use for detecting sperm DNA fragmentation in male infertility and comparison with other techniques. J Androl 23,25–43.

Filatov MV, Semenova EV, Vorob'eva OA, Leont'eva OA and Drobchenko EA (1999) Relationship between abnormal sperm chromatin packing and IVF results. Mol Hum Reprod 5,825–830.

Gandini L, Lombardo F, Paoli D, Caponecchia L, Familiari G, Verlengia C, Dondero F and Lenzi A (2000) Study of apoptotic DNA fragmentation in human spermatozoa. Hum Reprod 15,830–839.

Hacker-Klom UB, Göhde W, Nieschlag, E and Behre HM (1999) DNA flow cytometry of human semen. Hum Reprod 14,2506–2512.

Hammadeh ME, StieberM, Haidl G and Schmidt W (1998) Association between sperm cell chromatin condensation, morphology based on strict criteria, and fertilization, cleavage and pregnancy rates in an IVF program. Andrologia 30,29–35.

Hammadeh ME, Al-Sahani S, Doerr S, Stieber M, Rosenbaum P, Schmidt W and Diedrich K (1999) Comparison between chromatin condensation and morphology from testis biopsy extracted and ejaculated spermatozoa and their relationship to ICSI outcome. Hum Reprod 14,363–367.

Hammadeh ME, Strehler E, Zeginiadou T, Rosenbaum P and Schmidt W (2001a) Chromatin decondensation of human sperm in vitro and its relation to fertilization rate after ICSI. Arch Androl 47,83–87.

Hammadeh ME, Zeginiadov T, Rosenbaum P, Georg T, Schmidt W and Strehler E (2001b) Predictive value of sperm chromatin condensation (aniline blue staining) in the assessment of male fertility. Arch Androl 46,99–104.

Hansen M, Kurinczuk JJ, Bower, C and Webb S (2002) The risk of major birth defects after intracytoplasmic sperm injection and in vitro fertilization. N Engl J Med 346,725–730.

Hewitson L, Simerly CR and Schatten G (2003) ICSI, male pronuclear remodeling and cell cycle checkpoints. Adv Exp Med Biol 518,199–210.

Hoshi K, Katayose H, Yanagida K, Kimura Y and Sato A (1996) The relationship between acridine orange fluorescence of sperm nuclei and the fertilizing ability of human sperm. Fertil Steril 66,634–639.

Host E, Lindenberg S and Smidt-Jensen S (2000) The role of DNA strand breaks in human spermatozoa used for IVF and ICSI. Acta Obstet Gynecol Scand 79,559–563.

Host E, Ernst E, Lindenberg S and Smidt-Jensen S (2001) Morphology of spermatozoa used in IVF and ICSI from oligozoospermic men. Reprod Biomed Online 3,212–215.

Irvine DS, Twigg JP, Gordon EL, Fulton N, Milne PA and Aitken RJ (2000) DNA integrity in human spermatozoa: relationships with semen quality. J Androl 21,33–44.

Katayose H, Yanagida K, Hashimoto S, Yamada H and Sato A (2003) Use of diamide-acridine orange fluorescence staining to detect aberrant protamination of human-ejaculated sperm nuclei. Fertil Steril 79 (Suppl 1),670–676.

Katz P, Nachtigall R and Showstack J (2002) The economic impact of the assisted reproductive technologies. Nature Cell Biol 4 (Suppl),s29–s32.

Larson KL, DeJonge CJ, Barnes AM, Jost LK and Evenson DP (2000) Sperm chromatin structure assay parameters as predictors of failed pregnancy following assisted reproductive techniques. Hum Reprod 15,1717–1722.

Larson-Cook KL, Brannian JD, Hansen KA, Kasperson KM, Aamold ET and

Evenson DP (2003) Relationship between the outcomes of assisted reproductive techniques and sperm DNA fragmentation as measured by the sperm chromatin structure assay. Fertil Steril 80,895–902.

Lopes S, Sun JG, Jurisicova A, Meriano J and Casper RF (1998) Sperm deoxyribonucleic acid fragmentation is increased in poor-quality semen samples and correlates with failed fertilization in intracytoplasmic sperm injection. Fertil Steril 69,528–532.

Macas E, Imthurn B, Rosselli M and Keller PJ (1996) The chromosomal complements of multipronuclear human zygotes resulting from intracytoplasmic sperm injection. Hum Reprod 11,2496–2501.

Mansour RT, Fahmy IM, Taha AK, Tawab NA, Serour GI and Aboulghar MA (2003) Intracytoplasmic spermatid injection can result in the delivery of normal offspring. J Androl 24,757–764.

Moll AC, Imhof SM, Cruysberg JR, Schouten-van Meeteren AY, Boers M and van Leeuwen FE (2003) Incidence of retinoblastoma in children born after in-vitro fertilisation. Lancet 361,309–310.

Morris ID, Ilott S, Dixon L and 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. Hum Reprod 17,990–998.

Muratori M, Maggi M, Spinelli S, Filimberti E, Forti G and Baldi E (2003) Spontaneous DNA fragmentation in swim-up selected human spermatozoa during long term incubation. J Androl 24,253–262.

Orstavik KH, Eiklid K, van der Hagen CB, Spetalen S, Kierulf K, Skjeldal O and Buiting K (2003) Another case of imprinting defect in a girl with Angelman syndrome who was conceived by intracytoplasmic semen injection. Am J Hum Genet 72,218–219.

Perreault SD, Aitken RJ, Baker HW, Evenson DP, Huszar G, Irvine DS, Morris ID, Morris RA, Robbins WA, Sakkas D, Spanò M and Wyrobek AJ (2003) Integrating new tests of sperm genetic integrity into semen analysis: breakout group discussion. Adv Exp Med Biol 518,253–268.

Razavi S, Nasr-Esfahani MH, Mardani M, Mafi A and Moghdam A (2003) Effect of human sperm chromatin anomalies on fertilization outcome post-ICSI. Andrologia 35,238–243.

Retzloff MG and Hornstein MD (2003) Is intracytoplasmic sperm injection safe? Fertil Steril 80,851–859.

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

Sakkas D, Urner F, Bizzaro D, Manicardi G, Bianchi PG, Shoukir Y and Campana A (1998) Sperm nuclear DNA damage and altered chromatin structure: effect on fertilization and embryo development. Hum Reprod 13 (Suppl 4),11–19.

Sakkas D, Manicardi GC, Tomlinson M, Mandrioli M, Bizzaro D, Bianchi PG and Bianchi U (2000a) The use of two density gradient centrifugation techniques and the swim-up method to separate spermatozoa with chromatin and nuclear DNA anomalies. Hum Reprod 15,1112–1116.

Sakkas D, Manicardi G, Bizzaro D and Bianchi PG (2000b) Possible consequences of performing intracytoplasmic sperm injection (ICSI) with sperm possessing nuclear DNA damage. Hum Fertil (Camb) 3,26–30.

Sakkas D, Moffatt O, Manicardi GC, Mariethoz E, Tarozzi N and Bizzaro D (2002) Nature of DNA damage in ejaculated human spermatozoa and the possible involvement of apoptosis. Biol Reprod 66,1061–1067.

Sakkas D, Manicardi GC and Bizzaro D (2003) Sperm nuclear DNA damage in the human. Adv Exp Med Biol 518,73–84.

Saleh RA, Agarwal A, Nelson DR, Nada EA, El-Tonsy MH, Alvarez JG, Thomas AJ and Sharma RK (2002) Increased sperm nuclear DNA damage in normozoospermic infertile men: a prospective study. Fertil Steril 78,313–318.

Saleh RA, Agarwal A, Nada EA, El-Tonsy MH, Sharma RK, Meyer A, Nelson DR and Thomas AJ (2003) Negative effects of increased sperm DNA damage in relation to seminal oxidative stress in men with idiopathic and male factor infertility. Fertil Steril 79 (Suppl. 3),1597–1605.

Sanchez R, Stalf T, Khanaga O, Turley H, Gips H, Schill WB (1996) Sperm selection methods for intracytoplasmatic sperm injection (ICSI) in andrological patients. J Assist Reprod Genet 13,228–233.

Schieve LA, Meikle SF, Ferre C, Peterson HB, Jeng G and Wilcox LS (2002) Low and very low birth weight in infants conceived with use of assisted reproductive technology. N Engl J Med 346,731–737.

Schmid TE, Kamischke A, Bollwein H, Nieschlag E and Brinkworth MH (2003) Genetic damage in oligozoospermic patients detected by fluorescence in-situ hybridization, inverse restriction site mutation assay, sperm chromatin structure assay and the Comet assay. Hum Reprod 18,1474–1480.

Schultz RM and Williams CJ (2002) The science of ART. Science 296,2188–2190.

Sorahan T, Prior P, Lancashire RJ, Faux SP, Hulten MA, Peck IM and Stewart AM (1997) Childhood cancer and parental use of tobacco: deaths from 1971 to 1976. Br J Cancer 76,1525–1531

Spanò M, Cordelli E, Leter G, Lombardo F, Lenzi A and Gandini L (1999) Nuclear chromatin variations in human spermatozoa undergoing swim-up and cryopreservation evaluated by the flow cytometric sperm chromatin structure assay. Mol Hum Reprod 5,29–37.

Spanò M, Bonde JP, Hjøllund HI, Kolstad HA, Cordelli E, Leter G and The Danish First Pregnancy Planner Study Team (2000) Sperm chromatin damage impairs human fertility. Fertil Steril 73,43–50.

Stromberg B, Dahlquist G, Ericson A, Finnstrom O, Koster M and Stjernqvist K (2002) Neurological sequelae in children born after in-vitro fertilisation: a population-based study. Lancet 359,461–465.

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

Tejada RI, Mitchell JC, Norman A, Marik JJ and Friedman SA (1984) A test for the practical evaluation of male fertility by acridine orange. Fertil Steril 42,87–91.

Terada Y, Nakamura S, Morita J, Simerly C, Hewitson L, Murakami T, Yaegashi N, Schatten G and Okamura K (2003) Intracytoplasmic sperm injection: stiletto conception or a stab in the dark. Arch Androl 49,169–177.

Tomlinson MJ, Moffatt O, Manicardi GC, Bizzaro D, Afnan M and Sakkas D (2001) Interrelationships between seminal parameters and sperm nuclear DNA damage before and after density gradient centrifugation: implications for assisted conception. Hum Reprod 16,2160–2165.

Tomsu M, Sharma V and Miller D (2002) Embryo quality and IVF treatment outcomes may correlate with different sperm comet assay parameters. Hum Reprod 17,1856–1862.

Tournaye H (2003) ICSI: a technique too far? Int J Androl 26,63–69.

Twigg JP, Irvine DS and Aitken RJ (1998) Oxidative damage to DNA human spermatozoa does not preclude the pronucleus formation at intracytoplasmic sperm injection. Hum Reprod 13,1864–1871.

Veeck L (1991) Atlas of the human oocyte and early conceptus. Lippincott Williams and Wilkins, Baltimore.

Virant-Klun I, Tomazevic T and Meden-Vrtovec H. (2002) Sperm single-stranded DNA, detected by acridine orange staining, reduces fertilization and quality of ICSI-derived embryos. J Assist Reprod Genet 19,319–328.

World Health Organization. (1999) WHO Laboratory Manual for the Examination of Human Semen and Sperm–Cervical Mucus Interaction, 4th edn. Cambridge University Press,

Zini A, Finelli A, Phang D and Jarvi K (2000a) Influence of semen processing technique on human sperm DNA integrity. Urology 56,1081–1084.

Zini A, Nam RK, Mak V, Phang D and Jarvi K (2000b) Influence of initial semen quality on the integrity of human sperm DNA following semen processing. Fertil Steril 74,824–827.

Zini A, Bielecki R, Phang D and Zenzes MT (2001a) Correlations between two markers of sperm DNA integrity, DNA denaturation and DNA fragmentation, in fertile and infertile men. Fertil Steril 75,674–677.

Zini A, Kamal K, Phang D, Willis J and Jarvi K (2001b) Biologic variability of sperm DNA denaturation in infertile men. Urology 58, 258–261.