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Fall in implantation rates following ICSI with sperm with high DNA fragmentation

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BACKGROUND: There is considerable uncertainty as to the significance of a high sperm DNA fragmentation index (DFI) for achieving a successful pregnancy.

METHODS: The sperm DFI of 124 patients undergoing 192 IVF cycles and of 96 patients undergoing 155 ICSI cycles was determined using the sperm chromatin structure assay on neat sperm.

RESULTS: The rate of continuing pregnancies in ICSI cycles (but not in IVF cycles) showed significant negative correlation ($r = -0.184$, $P = 0.022$) with the DFI value. A threshold value of DFI which showed a significant difference ($P = 0.005$) in rate of continuing pregnancies between higher and lower DFI levels was found for ICSI cycles to be $\geq$19%, but no such threshold was found for IVF cycles. However, if the threshold of $\geq$30% was used for IVF cycles there was a non-significant lowering of the rates of continuing pregnancy and implantation at the higher DFI levels. DFI level had no effect on fertilization rate or on the percentage of embryos having more than 4 cells at Day 3 after fertilization. A high DFI level had a marked significant effect ($P = 0.001$) on implantation rate in ICSI cycles but not in IVF cycles. A significant positive correlation ($r = 0.268$, $P = 0.001$) between DFI and sperm midpiece defects was also noted in the ICSI patients.

CONCLUSIONS: These observations may help to resolve the issues about how, and to what extent, sperm DNA damage impacts upon the success of IVF and ICSI procedures.

Key words: DNA damage / implantation / midpiece defects / IVF/ICSI outcome / male infertility

Introduction

A number of tests have been developed to measure the proportion of strand breaks in the DNA of mammalian spermatozoa. The sperm chromatin structure assay (SCSA; Evenson and Jost, 1994; Evenson *et al.*, 2002) measures the susceptibility of DNA to acid-induced denaturation. The method uses acridine orange (AO), which gives green fluorescence with native DNA and red fluorescence with single-stranded DNA. The proportion of red fluorescence to total fluorescence in each sperm cell is measured by flow cytometry. The percentage of cells exhibiting a high proportion of red fluorescence was originally known as COMPα_t (Cells Outside the Main Population), but is now represented by the DNA fragmentation index (DFI). The method is recognized as being robust and reproducible, and has been used for some years in animal husbandry and human fertility studies (Ballachey *et al.*, 1987; Evenson *et al.*, 1999). Another much-used method of measuring sperm DNA fragmentation is the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay using flow cytometry or fluorescence microscopy. The TUNEL assay identifies DNA breaks by labelling 3′OH termini and is a measure of existing DNA damage, whereas the SCSA measures single-stranded DNA after acid treatment and therefore includes potential DNA damage.

It was initially hoped that the SCSA would supplement assays in routine use in IVF clinics, such as those measuring sperm concentration, motility and morphology, and would give a reliable and independent measure of fertility. Population studies of fecundity (Evenson *et al.*, 1999; Spano *et al.*, 2000) and of the outcomes of intrauterine insemination (Bungum *et al.*, 2007) have shown that pregnancy outcome is inversely related to the DFI. However, the effect of raised DFI upon the outcomes of IVF and ICSI is more controversial. Several authors have observed, using SCSA (Larson-Cook *et al.*, 2003;

Virro et al., 2004; Adams et al., 2004), or using TUNEL (Henkel et al., 2003; Frydman et al., 2008) that raised DNA fragmentation predicates a significantly lower pregnancy rate from IVF and/or ICSI treatment.

However, many authors have found no significant relationship between DFI and pregnancy outcome from IVF or ICSI (Bungum et al., 2004, 2007; Gandini et al., 2004; Gardner et al., 2004; Check et al., 2005; Payne et al., 2005; Zini et al., 2005; Caglar et al., 2007; Lin et al., 2008; Nicopoullos et al., 2008). It has also emerged that pregnancies can ensue when the male has DFI levels exceeding 27–30% (Gandini et al., 2004; Check et al., 2005; Boe-Hansen et al., 2006; Lin et al., 2008). Originally 30% was proposed as a threshold level above which pregnancy was very unlikely to occur.

The lack of consistency in the findings of different investigators has resulted in considerable uncertainty as to the significance of high DFI. The importance of the problem to the counselling and treatment of infertile patients is reflected in the number of meta-analyses that have been carried out. Both Collins et al. (2008) and the Practice Committee of the American Society for Reproductive Medicine (2006) concluded that tests for DNA damage were insufficiently predictive of pregnancy outcome to justify their routine use in the evaluation of infertility. Evenson and Wixon (2006) concluded from a meta-analysis that a high DFI has less effect on the success of ICSI cycles than on IVF cycles, and suggested that high DFI patients should be directed towards ICSI in preference to IVF. The meta-analysis of Li et al. (2006) indicated that results of the TUNEL assay showed a significant effect of high DFI on pregnancy outcome for IVF but not ICSI, although the results of the SCSA assay failed to show a significant effect on either IVF or ICSI outcomes. These meta-analyses point out the need for further research (Zini and Libman, 2006; Tarozzi et al., 2007).

In the present study, we sought to establish firstly whether high DFI has any effect on pregnancy outcome, and secondly if so, at which points in the IVF or ICSI cycle the adverse effects operate.

Materials and Methods

Patients

The study was approved by the University College London Ethics Committee, UCLH/UCL R&D Governance and UCL Data Protection. Ninety-five patients who underwent routine semen analysis at the Centre for Reproductive and Genetic Health (CRGH) of University College London Hospital, gave written consent and later went on to have one or more IVF or ICSI cycles were recruited into the study. The semen was obtained by ejaculation. After the routine diagnostic semen analysis had been performed, a portion of the neat semen was divided into aliquots and stored at −80°C until used for the SCSA assay, which was performed in-house.

A further group of 125 patients who had experienced one or more IVF or ICSI cycles, and whose semen had undergone an SCSA assay at the private clinic The Doctors' Laboratory between 2004 and 2009 were also included in the study. These patients had infertility linked to male factor or had experienced at least two failed cycles of assisted reproduction. For some patients, SCSA assays were performed by both clinics, with the semen collected on different days and the results were comparable ($r = 0.844$, $P < 0.001$, $n = 11$). Therefore, the results from the two clinics are grouped together.

Patients found to have an abnormal blood karyotype were excluded from the study. All female patients were <45 years of age. Only IVF and ICSI cycles are reported. Thus frozen/thawed embryo transfers and intrauterine inseminations are excluded. Also excluded are cycles where embryos were produced but for various reasons were not transferred as fresh embryos in that cycle. A total of 347 cycles met the above criteria. Table I shows clinical information on the patients included in the study, together with details on their 192 IVF and 155 ICSI cycles and on their semen samples.

IVF and ICSI procedures

The stimulation protocol was individualized after evaluation of the ovarian reserve by antral follicle count, FSH, estradiol and anti-Mullerian hormone. A long down-regulation protocol was used for patients with normal ovarian reserve. Patients with reduced reserve were stimulated with antagonist drug regimen and high starting dose of gonadotrophins. Follicular growth was monitored by ultrasound scans from Day 7 of stimulation. When at least two leading follicles reached the diameter of 18 mm hCG was administered. Ultrasound guided vaginal oocyte retrieval was performed at 37 h post-hCG injection. IVF or ICSI was performed at 40 and 41 h post-hCG, respectively. ICSI was the procedure used when semen quality was poor (e.g. low concentration and/or poor motility), when IVF had led to poor fertilization rate, and also in seven cycles of PGD needing DNA amplification where using IVF might have led to contamination. Fertilization was evaluated at 18–20 h post-insemination. Embryos were cultured in IVF medium (GIII series, Vitrolife, UK).

Sperm chromatin structure assay

The in-house SCSA assay followed the procedure previously described elsewhere (Evenson and Jost, 1994; Evenson et al., 2002). A 100–200 µl aliquot of frozen neat semen was rapidly thawed at 37°C, then diluted with a suitable volume (depending on the sperm concentration) of TNE buffer (150 mM NaCl, 10 mM Tris–HCl and 1.0 mM disodium EDTA, pH 7.4). A total of $1–2 \times 10^6$ sperm cells were treated according to Evenson et al. (2002). The AO reagent used in this assay was molecular biology grade, Cat. No. A6014, Sigma-Aldrich Company Ltd, Poole, Dorset, UK. This AO was found to give the same results ($r = 0.999$, $P < 0.001$) as the Polysciences AO recommended by Evenson et al. (2002). Flow cytometry was performed on a Beckman-Coulter Epics Elite cytometer (High Wycombe, UK; Fig. 1). A number of samples were analysed on two other flow cytometers. The relevant populations were found to be better separated using the Beckman-Coulter instrument, which was therefore used throughout the study.

Statistical analysis

The Statistical Package for the Social Sciences (SPSS) version 14.0 for Windows was used for Pearson correlations. Comparison of means used the Student's *t*-test for independent samples (SPSS and Excel). Statistical significance was set at $P < 0.05$.

Results

As expected, male factor was the main cause of infertility in patients treated by ICSI but not in those treated by IVF ($P < 0.001$). For ICSI patients, tubal disease and 'unexplained' were much less commonly named as cause of infertility ($P < 0.001$ for both classifications). The ICSI patients also had a lower average sperm concentration, motility and normal forms than patients treated by IVF ($P < 0.001$ for each of the three sperm properties). In the cycles, the number of 2PN embryos ($P < 0.001$), morulas ($P = 0.018$) and blastocysts ($P = 0.008$) per treatment cycle was also lower in the patients treated by ICSI versus IVF.

Table I Clinical data on patients whose cycles proceeded to embryo transfer.

	IVF treatment	ICSI treatment	*P*
(a) Data on patients			
Number of patients	124	96	
Average female age (years ± SD)	36.4 (±3.95)	35.2 (±3.85)	0.031*
Average male age (years ± SD)	37.8 (±5.18)	37.8 (±6.06)	NS
Diagnosis	% of patients	% of patients	
Male factor	7.3	79.2	<0.001*
Tubal disease	20.2	2.1	<0.001*
Endometriosis	6.5	2.1	NS
Ovulatory disorder	5.6	1.0	NS
Polycystic ovaries	3.2	1.0	NS
Uterine fibroids	0	1.0	NS
Unexplained	57.3	8.3	<0.001*
DNA amplification requiring ICSI	0	5.2	In method
(b) Data on sperm (1 semen sample per patient)			
Mean sperm concentration (million/ml)	35.7	14.4	<0.001*
Mean percentage motility (%)	57.9	42.3	<0.001*
Mean normal forms (%)	32.9	24.8	<0.001*
Patients with $>1 \times 10^6$/ml round cells (%)	15.3	26.0	0.049*
Patients with $>1 \times 10^6$/ml leucocytes (%)	1.6	0	NS
(c) Data on cycles (1–4 cycles per patient)			
Number of cycles	192	155	
Mean no. of 2PN embryos per cycle	8.35	6.45	<0.001*
Mean no. of Day 5 morulas per cycle	1.96	1.26	0.018*
Mean no. of Day 5 blastocysts per cycle	1.12	0.63	0.008*
Mean no. of embryos transferred	1.97	1.93	NS
Implantation rate/embryo transfer (%)	21.6	19.6	NS
Live birth events (% of cycles)	25.5	25.2	NS
Percentage of birth events that were twin	26.5	23.1	NS
Mean duration of pregnancy (singleton) (weeks)	38.6	39.7	NS
Mean duration of pregnancy (twin) (weeks)	35.5	36.3	NS
Mean birthweight (singleton births) (g)	3124	3336	NS
Mean birthweight (twin births) (g)	2280	2492	NS

*Significant difference: IVF versus ICSI.
2PN, 2 pronuclei.

Effect of DFI on pregnancy outcome

In this paper, continuing pregnancy means a pregnancy which resulted in a live birth event, unless otherwise stated. A live birth event means either a single or a multiple birth.

A simple plot of DFI against positive or negative pregnancy for each cycle produced a graph where multiple points overlapped, so the cycles (IVF and ICSI together) were arranged in order of increasing DFI, cut into groups of 28–29 cycles and mean DFI of each group was plotted against mean percentage of continuing pregnancies for that group (Fig. 2). The graph demonstrates a significant ($P = 0.002$) negative correlation ($r = -0.800$) between DFI and continuing pregnancy.

Table II shows Pearson correlation coefficients and significance levels for pregnancy outcomes in the 192 IVF and 155 ICSI cycles studied. ICSI cycles showed significant correlation between DFI and continuing pregnancies and between DFI and no pregnancy, but not between DFI and miscarriages. In the case of IVF cycles there was no significant correlation between DFI and pregnancy outcome.

We examined the effect of varying the DFI threshold value on the ability of the SCSA to predict pregnancy outcome in IVF and ICSI cycles separately. Although including all the cycles for each patient gives the maximum information, an alternative is to consider only the first cycle of each patient and Table III shows both types of display. Different values of DFI were taken as proposed threshold levels and the *P*-value of difference between the means was calculated for each. For IVF cycles, significance was not attained for any threshold

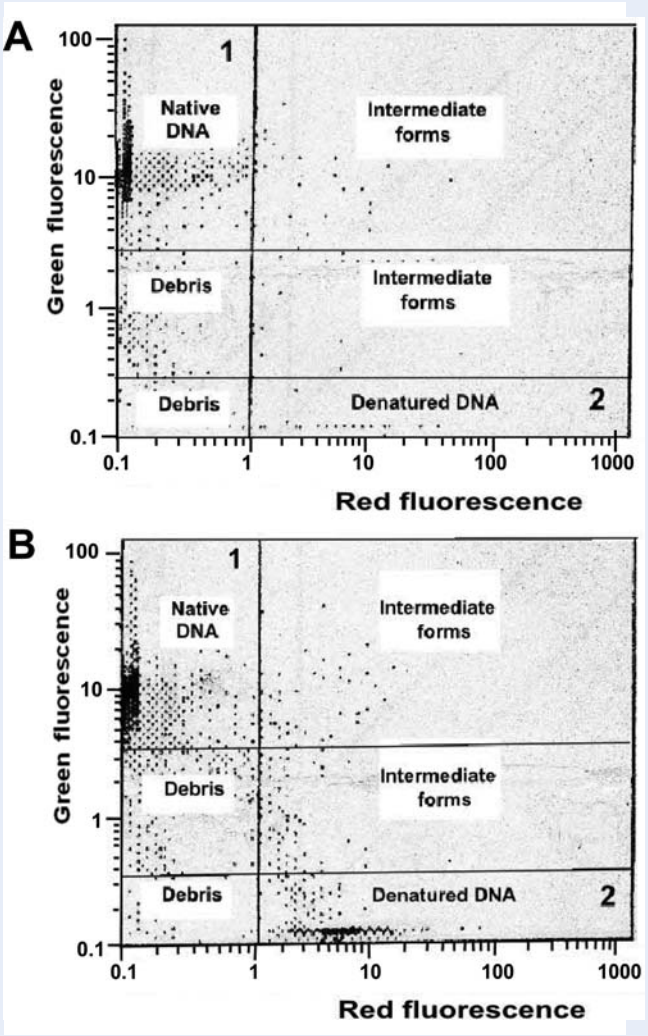

Figure 1 Print-out from the Beckman-Coulter Epics Elite flow cytometer.
The different sperm populations show good separation. The DFI was calculated as the counts in Box 2 as a percentage of the sum of the counts in Boxes 1 and 2. (**A**) DFI = 1%; (**B**) DFI = 28%.

values. For ICSI cycles, a threshold value of ≥18–19% showed the highest level of significance ($P = 0.001–0.005$).

Table IV shows continuing pregnancy, no pregnancy and miscarriage etc. rates when the threshold values ≥19% and ≥30% are used for IVF and ICSI cycles separately. The table uses all the cycles for each patient. The threshold of ≥30% is included in the table because there is some indication in Table III of a minimum value of P in this region for all IVF cycles. It is seen that for ICSI cycles using the threshold ≥19% there are significant differences between the upper and lower DFI ranges for the rate of continuing pregnancies and the rate of non-pregnancy but not for the rate of miscarriages. For IVF cycles there is a significant difference in non-pregnancy rate between the two DFI ranges, and the continuing pregnancy rate is markedly (although non-significantly) lower in the higher DFI range. This justifies the continued use of ≥30% as a possible threshold level for IVF cycles.

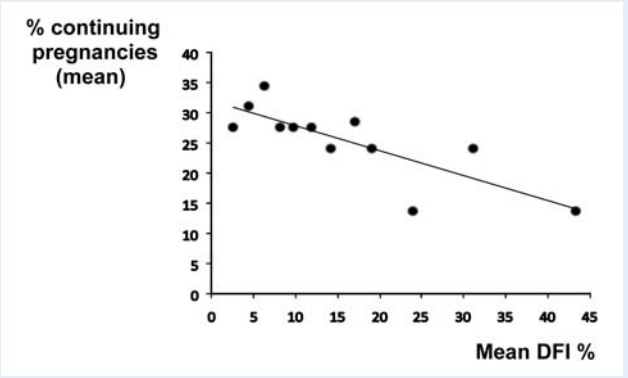

Figure 2 The effect of DFI on the percentage of live birth events. The 347 IVF and ICSI cycles were arranged in order of increasing DFI, then split into groups of 28 or 29 cycles. The mean DFI of each group was plotted against mean percentage of live birth events. Correlation coefficient $r = -0.800$; $P = 0.002$.

Effect of DFI on fertilization rate and number of cells per embryo at Day 3

(i) The fertilization rate for each treatment cycle was calculated as the number of embryos with 2PN as a percentage of the number of oocytes inseminated or injected.
(ii) To gain an estimate of the growth of embryos between fertilization and Day 3, the number of embryos at Day 3 of the cycle having more than 4 cells per embryo was calculated as a percentage of the number of initial 2PN embryos.

The two properties were investigated by the same statistical methods. First the Pearson correlation test was performed to see if there was any correlation between the fertilization rate and DFI value or between the growth of embryos and DFI value. This was carried out for IVF cycles and ICSI cycles separately. No significant correlation was seen. Next the *t*-test was carried out to look for any DFI threshold value which showed a significant negative effect of higher DFI upon the fertilization or growth, but none was found. It was concluded that DFI value did not negatively affect fertilization or growth up to Day 3 after fertilization.

Effect of DFI on percentage of morulas and blastocysts at Day 5 after fertilization

Table V shows a possible tendency for fewer blastocysts (but not morulas) in the higher DFI range, but this did not reach significance.

Effect of DFI on implantation rate

Because of the threshold values shown in Table III of DFI ≥ 19% (for ICSI), and a tendency to a minimum *P* at ≥30% (for IVF), implantation rates for IVF and ICSI cycles for these two thresholds are given in Table VI. For IVF alone, using the DFI threshold value of ≥30% led to a 48% decrease in implantation rate in the higher DFI range, but this did not reach significance. For ICSI alone, using the DFI threshold value of ≥19% led to a highly significant difference ($P = 0.001$) in implantation rate between the higher and lower DFI ranges (When the threshold was lowered to DFI ≥ 17% and ≥16% the significance *P* became less marked at 0.009 and 0.067, respectively).

Table II Pearson correlation coefficients (*r*) and significance levels (*P*) for DFI % against pregnancy outcome (%).

	Number of		Continuing pregnancies[a]		No pregnancy		Miscarriages etc.[b]	
	Patients	Cycles	*r*	*P*	*r*	*P*	*r*	*P*
IVF cycles	124	192	−0.054	0.453	0.129	0.074	−0.122	0.091
ICSI cycles	96	155	−0.184*	0.022*	0.197*	0.014*	−0.048	0.555

*Significant.
[a]Continuing pregnancies have all now resulted in live births apart from one at 6 months still continuing.
[b]Miscarriages etc. comprise miscarriages, biochemical pregnancies and terminations. A positive correlation between DFI and miscarriage rate would result in a positive figure for r in addition to a low figure for *P*.

Table III *P*-values of differences between the means, calculated from the data for DFI versus continuing pregnancy, taking different values of DFI as the threshold value.

IVF cycles (all cycles) (*n* = 192)		IVF cycles (1 per patient) (*n* = 124)		ICSI cycles (all cycles) (*n* = 155)		ICSI cycles (1 per patient) (*n* = 96)	
Threshold DFI %	*P*-value	Threshold DFI %	*P*-value	Threshold DFI %	*P*-value	Threshold DFI %	*P*-value
≥44	0.615	≥44	0.765	≥31	0.633	≥31	0.575
≥38	0.250	≥38	0.970	≥30	0.490	≥30	0.420
≥37	0.568	≥37	0.463	≥27	0.319	≥27	0.298
≥33	0.262	≥33	0.865	≥25	0.086	≥25	0.070
≥32	0.214	≥32	0.957	≥23	0.038	≥23	0.043
≥31	0.214	≥31	0.957	≥21	0.022	≥21	0.030
≥30	0.214	≥30	0.957	≥20	0.013	≥20	0.022
≥27	0.474	≥27	0.665	→ ≥19	0.005	≥19	0.006
≥24	0.853	≥24	0.947	≥18	0.005	→ ≥18	0.001
≥20	0.520	≥20	0.587	≥17	0.027	≥17	0.007
≥19	0.801	≥19	0.838	≥16	0.109	≥16	0.052
≥14	0.640	≥14	0.988	≥14	0.050	≥14	0.006
≥8	0.714	≥8	0.798	≥8	0.111	≥8	0.041

Arrows (→) indicate DFI values which show a relatively high level of significance and are therefore suitable as threshold values.

This result indicates that the sperm used for ICSI having a DFI ≥ 19% was associated with a lower implantation rate than the sperm used for ICSI having a DFI < 19%.

Comparison of clinical details above and below DFI threshold levels

To further investigate the effect of DFI upon implantation rate, clinical details of patients were compared above and below threshold levels (Table VII). In ICSI cycles the average age above and below the threshold level was similar. The percentage of male factor infertility was also similar, which is explained by this being the predominant reason for choosing ICSI treatment. Of the sperm properties, the percentage of normal forms was significantly different in the ICSI cycles, and the total motility, i.e. the percentage of sperm counted as motile, was significantly different above and below threshold values in both IVF and ICSI cycles.

Table VIII shows a further study of these properties by Pearson correlation. This statistical method has the advantage that no assumptions with regard to threshold levels need be made. The third section of the table includes all the patients for whom the DFI value was known, whether or not they proceeded to IVF or ICSI treatment. With this larger number of patients a strong correlation of DFI with male age, sperm concentration and total motility is seen, and a less strong but still significant correlation with normal forms. It was of interest to know what part of the sperm morphology (seen by light microscopy) was most affected by high DFI. The only evidence seen was a very strong correlation between DFI and midpiece defects in ICSI cycles, but this was not seen in IVF cycles or in the larger group of patients. Using comparison of means the effect in ICSI cycles was most significant around DFI ≥ 34%. No significant correlation between DFI and head or tail defects was seen in any of the patient groups.

Table IV Comparison of reproductive outcomes for patients using DFI threshold values ≤19 and ≤30%.

	IVF cycles					ICSI cycles				
	Patients (*n*)	Cycles (*n*)	Continuing preg.	No preg.	Miscarriages etc.[a]	Patients (*n*)	Cycles (*n*)	Continuing preg.	No preg.	Miscarriages etc.[a]
DFI ≥ 19%	18	33	27.3	69.7	3.0	44	74	14.9	75.7	9.5
DFI < 19%	106	159	25.2	61.6	13.2	52	81	34.6	55.6	9.9
P			0.801	0.422	0.096			0.005*	0.008*	0.931
DFI ≥ 30%	8	16	12.5	87.5	0.0	22	34	20.6	70.6	8.8
DFI < 30%	116	176	26.7	60.8	12.5	74	121	26.4	63.6	9.9
P			0.214	0.038*	0.134			0.490	0.455	0.850

The figures are % of cycles.
*Significant.
[a]Miscarriages etc. comprised pregnancies failing to result in live birth for any reason. A biochemical pregnancy was defined as β-HCG > 50 IU/ml but no fetal heart seen. In IVF cycles there were 10 biochemical pregnancies, 10 miscarriages, 1 medical termination and 1 ectopic pregnancy, all occurring with DFI < 30. In ICSI cycles there were 11 biochemical pregnancies (5 with DFI ≥ 19%), 2 miscarriages (1 with DFI ≥ 19%), 1 ectopic pregnancy (DFI = 16) and 1 medical termination (DFI = 21).

Discussion

The investigation reported here was carried out in a clinical setting, and therefore the group of patients having ICSI cycles differed from the group having IVF cycles in that the infertility the ICSI group was experiencing was predominantly associated with male factors. One aim of this study was to discover whether couples where the male has a high DFI have a significantly lower rate of continuing pregnancies in IVF and ICSI cycles than couples with a lower DFI. The statistical evidence shown in Tables II–IV supports this for ICSI cycles. The evidence for the IVF cycles was less strong, showing a non-significant trend towards lower continuing pregnancy and implantation rates at DFI levels ≥30%. The IVF cycles had a lower percentage of male factor infertility and a lower proportion of high DFI patients than the ICSI cycles (Tables I and IV). The greater effect of high DFI upon ICSI cycles compared with IVF cycles is supported by Benchaib *et al.* (2003) who found a lack of correlation between pregnancy rate and DFI in 50 IVF patients, whereas there was a correlation $P < 0.05$ with ICSI in 54 patients. Bakos *et al.* (2007) found a significant negative correlation ($P < 0.05$) between DFI and pregnancy outcome in 68 ICSI couples but not in 45 IVF couples. Borini *et al.* (2006) found a negative correlation ($P = 0.007$) between DFI and pregnancy rate in 50 ICSI patients, whereas in 82 IVF patients the correlation was non-significant ($P = 0.723$). However, Henkel *et al.* (2003) and Frydman *et al.* (2008) report a significant negative correlation in IVF cases between DFI and pregnancy rate.

In this type of study it is usual to choose a threshold level and to compare results above and below the threshold. Table III indicates that the threshold level for ICSI cycles in this study occurred at a markedly lower DFI level than that proposed (and commonly accepted) for IVF cycles using the SCSA method. This would explain the finding in the meta-analysis of Li *et al.* (2006) that, using SCSA, sperm DNA damage has no adverse effect on clinical pregnancy in either IVF or ICSI cycles. The three papers cited in the meta-analysis use different study designs, but the simplified account given in the meta-analysis states that Bungum *et al.* (2004) and Boe-Hansen *et al.* (2006) used a DFI cut-off value of 27% whereas Zini *et al.* (2005) used one of 30%. Neither of these values would have given a significant difference in continuing pregnancy rate for ICSI cycles in the present study since they lie well above the threshold of ≥19% used by us for these cycles.

The lower DFI threshold for ICSI cycles compared with IVF cycles indicates that the ICSI cycles (with their higher proportion of male factor infertility) are more sensitive to the harmful effects of DNA fragmentation, since the adverse effect on pregnancy rate occurs at a lower DFI level.

It is often stated that high DFI levels are associated with an increase in miscarriage rates: the present study found no evidence of an increase.

The next aim of the study was to investigate at which stage in the cycle the adverse effect of high DFI operates. When fertilization rates and number of cells per embryo at Day 3 after fertilization were examined, no effect of high DFI was seen. During the period up to Day 3 the embryo is considered to rely on maternal transcripts for its metabolism and growth (Tesarik *et al.*, 1986; Braude *et al.*, 1988). At Day 5, when the embryonic genome is participating in the metabolism of the embryo, there was a reduction in the number of blastocysts when DFI was in the upper range, but this did not reach levels of significance. Similarly Benchaib *et al.* (2003) and Seli *et al.*

Table V Percentages of embryos present as morulas and blastocysts at Day 5 after fertilization for patients using threshold DFI values ≥19 and ≥30%.

	IVF cycles				ICSI cycles				
	Patients (*n*)	Cycles (*n*)	Morulas (%)	Blastocysts (%)	Patients (*n*)	Cycles (*n*)	Morulas (%)	Blastocysts (%)	
DFI ≥ 19%	18	33	15.9	9.1	44	74	13.7	6.2	
DFI < 19%	106	159	18.8	11.2	52	81	17.3	10.0	
P			0.504	0.500				0.358	0.156
DFI ≥ 30%	8	16	17.9	9.3	22	34	17.0	5.9	
DFI < 30%	116	176	18.4	11.0	74	121	15.1	8.8	
P			0.938	0.704			0.697	0.369	

The values were calculated as the percentage of the original 2PN embryos which at Day 5 were present as morulas or blastocysts. They were calculated for each cycle, then those for each DFI range were averaged.

Table VI Implantation rates.

	IVF cycles				ICSI cycles			
	Patients (*n*)	Cycles (*n*)	Embryos (*n*)	Implantation rate (%)	Patients (*n*)	Cycles (*n*)	Embryos (*n*)	Implantation rate (%)
DFI ≥ 19%	17	32	68	22.1	44	74	149	12.1
DFI < 19%	104	157	311	21.5	51	80	147	27.2
P				0.926				0.001*
DFI ≥ 30%	8	16	34	11.8	22	34	70	17.2
DFI < 30%	113	173	345	22.6	73	120	226	20.4
P				0.144				0.556

Each transferred embryo that resulted in a fetal heart was expressed as 100%, and each that did not was expressed as 0%. These figures were then averaged for each DFI range. Four cycles produced no embryos and are omitted.
*Significance.

(2004) have observed a lowering of blastocyst formation with high DFI which did not reach significance. Virro *et al*. (2004) have reported a significant negative effect on the formation of blastocysts.

In ICSI cycles a very marked negative effect of high DFI upon implantation rate was observed, and in view of the lack of significant effect at previous stages this can be regarded as accounting for most of the adverse effect of high DFI upon continuing pregnancy rates. An effect on implantation has also been observed by Adams *et al*. (2004), Tesarik *et al*. (2004; Tesarik, 2005) and Frydman *et al*. (2008), and is termed the 'late paternal effect' by Tesarik *et al*. to distinguish it from other effects that occur earlier in the cycle and do not depend on DNA fragmentation.

The studies by previous workers reported above, supported by the present study, indicate clearly that strand breaks in the sperm DNA have little or no effect on fertilization and early embryo growth but begin to have an effect at the stage of blastocyst development, and then have a very marked effect on implantation of the embryo. There is also support from two studies in which sperm DNA was damaged by gamma irradiation and subsequently used in fertility studies (Ahmadi and Ng, 1999; Fatehi *et al*., 2006). There was no significant effect on fertilization rate or early cleavage, but the effects on blastocyst development and implantation rate were very marked.

The inverse relationship between DFI and sperm motility is well-established (Sun *et al*., 1997; Zini *et al*., 2001; Benchaib *et al*., 2003). This may contribute to the relative lack of significant negative correlation between high DFI and continuing pregnancy in IVF cycles compared with ICSI cycles. The spermatozoa with high DFI may lack certain properties required for IVF of an egg, the most obvious of which is motility, and in IVF cycles they will be less able to compete against any spermatozoa present in the same sample which possess relatively normal characteristics, resulting in a more normal spermatozoon fertilizing the egg. When doing ICSI, embryologists try to pick out the best spermatozoa, but some types of damage may not be seen. If better ways of selecting spermatozoa for ICSI, such as high magnification inspection of morphology (Bartoov *et al*., 2002) can be developed further, the disadvantage of ICSI in this respect may be lessened.

There was a very strong positive correlation between DFI and sperm midpiece defects in the sperm used for ICSI cycles. Midpiece defects were counted under the optical microscope as part of the routine sperm analysis and details of the defects were not recorded. They may, however, be related to the midpiece defects described by Said *et al*. (2005) which were found in the lighter fractions resulting from density gradient separation of semen from teratozoospermic

Table VII Comparison of certain parameters at DFI values above and below threshold values for all IVF and ICSI cycles.

	IVF cycles			ICSI cycles		
	DFI ≥ 30, *n* = 16	DFI < 30, *n* = 176	Significance, *P*	DFI ≥ 19, *n* = 74	DFI < 19, *n* = 81	Significance, *P*
Mean female age (years)	37.4, *n* = 16	36.5, *n* = 176	NS	35.6, *n* = 74	35.8, *n* = 81	NS
Mean male age (years)	42.6, *n* = 16	37.8, *n* = 176	0.001	38.9, *n* = 74	38.6, *n* = 81	NS
% of patients with male factor infertility	12.5, *n* = 16	6.3, *n* = 176	NS	81.1, *n* = 74	79.0, *n* = 81	NS
Sperm concentration (M/ml)	41.2, *n* = 16	35.9, *n* = 176	NS	11.7, *n* = 74	15.2, *n* = 81	NS
Sperm total motility (%)	51.9, *n* = 16	59.6, *n* = 176 176	0.036	39.1, *n* = 74	45.3, *n* = 81	0.026
Sperm normal forms (%)	35.1, *n* = 16	33.1 *n* = 176	NS	22.4, *n* = 70	27.6, *n* = 77	0.003

Some values for normal forms were not available.

Table VIII Pearson correlations of sperm parameters against DFI values for all IVF and ICSI cycles.

	IVF cycles			ICSI cycles			All patients		
	n	*r*	*P*	*n*	*r*	*P*	*n*	*r*	*P*
Male age (years)	192	0.270*	<0.001*	155	0.120	0.136	350	0.167*	0.002*
Concentration (M/ml)	192	0.056	0.439	155	−0.107	0.186	350	−0.219*	<0.001*
Total motility (%)	192	−0.241*	0.001*	155	−0.238*	0.003*	350	−0.391*	<0.001*
Normal forms (%)	192	0.092	0.206	147	−0.160	0.053	338	−0.124*	0.023*
Head defects (%)	191	0.078	0.284	144	−0.139	0.097	331	0.097	0.607
Midpiece defects (%)	191	−0.130	0.072	144	0.268*	0.001*	331	0.092	0.094
Tail defects (%)	191	−0.071	0.330	144	−0.016	0.845	331	0.012	0.833

The third section represents one sample from each of 350 patients (except where indicated) including those for IVF and ICSI treatment and also those patients not having either treatment.
Some values for morphology were not available.
*Significant.

patients. These lighter fractions contained more DNA strand breaks (measured by TUNEL) than the denser semen fractions and were active in generating reactive oxygen species. The midpiece defects in these fractions are considered to result from disordered spermiogenesis leading to enzyme-containing residual cytoplasm in the midpiece (Saleh and Agarwal, 2002). Midpiece defects of this type have aroused recent interest because of their possible role in initiating sperm DNA fragmentation (Aitken and De Iullis, 2010). On a practical note, it appears from our results that a finding of low motility combined with raised midpiece defects in a routine sperm analysis should lead one to suspect a high level of DNA fragmentation.

If the nuclear DNA in the spermatozoon used for ICSI is fragmented it is understandable that the complex process of implantation into the uterus is impaired, causing a lowering of the rate of continuing pregnancies in ICSI cycles when the DFI is ≥19%. In our unit, despite many severe cases of male factor infertility in the ICSI group, the average rate of continuing pregnancies for ICSI was the same as for IVF (Tables I and IV), and in the cycles with DFI ≥ 19% there were 11 live birth events resulting in 15 healthy children. Although the usefulness of ICSI remains beyond question, there is some concern that there may be long-term effects on offspring of using high DFI spermatozoa (Aitken and De Iullis, 2007). As long as this remains a possibility every effort should be made to find ways of lowering the DFI, such as by treatment with oral antioxidants (Greco *et al.*, 2005).

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