Human Reproduction Vol.21, No.11 pp. 2876–2881, 2006 doi:10.1093/humrep/del251
Advance Access publication June 22, 2006.

Sperm DNA fragmentation: paternal effect on early post-implantation embryo development in ART

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BACKGROUND: The relationship between early embryo post-implantation development in couples undergoing assisted reproductive techniques (ARTs) and sperm chromatin alterations has not been satisfactorily explained. The aim of this study was to assess the relationship between sperm DNA fragmentation in IVF/ICSI patients, sperm parameters (concentration, motility and morphology) and ART outcome, especially with regard to clinical pregnancy and pregnancy loss (spontaneous miscarriage or biochemical pregnancy). METHODS: DNA fragmentation was evaluated by TUNEL assay, performed on sperm suspensions after density gradient separation, in 132 men undergoing an ART cycle (82 IVF and 50 ICSI) and correlated with sperm parameters and ART outcome. RESULTS: A highly significant negative correlation was found between DNA fragmentation and sperm parameters. There was a close relationship between DNA fragmentation and post-implantation development in ICSI patients: the clinical pregnancy and pregnancy loss rates significantly differed between patients with high and low sperm DNA fragmentation ($P = 0.007$ and $P = 0.009$, respectively). CONCLUSIONS: Sperm DNA fragmentation seems to affect embryo post-implantation development in ICSI procedures: high sperm DNA fragmentation can compromise 'embryo viability', resulting in pregnancy loss.

Key words: abortion/ART/DNA fragmentation/human sperm/pregnancy

Introduction

Many studies have shown how a 'paternal effect' can cause repeated assisted reproductive technique (ART) failures (Vanderzwalmen *et al*., 1991; Perinaud *et al*., 1993; Janny and Menezo, 1994; Hammadeh *et al*., 1996; Sanchez *et al*., 1996; Shoukir *et al*., 1998). Many authors have shown how this 'paternal effect' can be traced back to anomalies in sperm chromatin organization: the sperm of subfertile men are characterized by susceptibility to acid-induced denaturation *in situ* (Spano *et al*., 2000), reduced chromatin condensation (Bianchi *et al*., 1996), chromosomic anomalies (Moosani *et al*., 1995) and/or increased DNA strand breaks (Lopes *et al*., 1998; Irvine *et al*., 2000). It has been shown how a high percentage of spermatozoa with alterations in chromatin structure has a negative effect on ART procedure outcome (Sun *et al*., 1997; Lopes *et al*., 1998; Larson *et al*., 2000; Morris *et al*., 2002; Benchaib *et al*., 2003). These studies have focused on testing for possible correlations between paternal chromatin alterations and fertilization, embryo cleavage, blastocyst development and clinical pregnancy rates, in both IVF and ICSI.

These findings suggest that paternal genomic alterations may compromise not only fertilization and embryo quality but also 'embryo viability' and progression of pregnancy, resulting in spontaneous miscarriage or biochemical pregnancy. To date, a number of studies in men and animals have highlighted the importance of the 'paternal factor', including male age or paternal exposure to toxic materials, in spontaneous miscarriages (Olshan and Faustman, 1993; Marchetti *et al*., 1999; Carrell and Liu, 2003; Carrell *et al*., 2003; Hjollund *et al*., 2005; Slama *et al*., 2005), but the relationship between 'early post-implantation embryo development' in couples undergoing ART and sperm DNA integrity still remains to be explained. The effect of altered sperm chromatin integrity on post-embryonic development is therefore still a matter of debate (Larson *et al*., 2000).

The aim of this study was to examine, in IVF and ICSI patients, the possible relationship between sperm DNA fragmentation, assessed by TUNEL assay, traditional sperm evaluation parameters (concentration, motility and morphology) and ART outcome, especially in regard to clinical pregnancy and, as a new issue, pregnancy loss, the latter defined as spontaneous miscarriage or biochemical pregnancy.

Subjects and methods

Patients

The study was carried out at Tecnobios Procreazione, Bologna, Italy. A total of 132 couples undergoing an ART cycle were included: 50 cycles were ICSI and 82 IVF. The mean age of women included in the

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study was 37.05 ± 4.19 years and the mean BMI was 22.17 ± 3.13 kg/m^2. The mean age of men was 40.24 ± 5.17 years. Only men with ejaculated sperm were included in the study.

The indication for ICSI treatment was severe male factor (sperm concentration $<10 \times 10^6$/ml and/or sperm motility <30% and/or normal sperm morphology <4%). This was associated with oligo-ovulation in 1 couple (2%), endometriosis in 5 couples (10%) and tubal defects in 10 couples (20%); aetiology of infertility was only male factor in 27 couples (54%) and unexplained in 7 couples (14%). In IVF patients, the aetiology of infertility was oligo-ovulation in 2 couples (2.4%), endometriosis in 14 couples (17.1%), unexplained in 17 couples (20.7%), tubal defects in 39 couples (47.6%) and male factor in 10 couples (12.2%).

Sperm collection and preparation

Sperm samples were collected by masturbation. Samples were analysed following WHO indications (WHO, 1999) for total sperm number, concentration, motility and morphology and were prepared using a discontinuous PureSperm gradient (Nidacon, Gothemberg, Sweden). Briefly, sperm was layered upon a 40:80% PureSperm density gradient, processed by centrifuge at $600 \times g$ for 15 min and resuspended in 1 ml of sperm culture medium (PureSperm wash, Nidacon, Gothemberg, Sweden). After density gradient separation, a second evaluation of concentration, motility and morphology was performed.

Ovarian stimulation, IVF, ICSI and embryo development

Ovarian stimulation was achieved by recombinant FSH (Gonal F, Serono, Rome, Italy; Puregon, Organon, Rome, Italy) and monitored by endovaginal echography and plasma estradiol. Thirty-six hours before oocyte retrieval, 10 000 IU of hCG (Gonasi, Amsa, Rome, Italy) was administered. Oocyte retrieval was carried out under general anaesthesia by a vaginal ultrasonography-guided aspiration. At 16–18 h after insemination or microinjection, as previously described (Borini *et al*., 2004a,b), oocytes were assessed for two PN presence. Forty-eight hours after oocyte retrieval, embryos were classified according to morphology and then transferred into the uterus. Clinical pregnancy was determined by ultrasound detection of gestational sac and biochemical pregnancy by the presence of only one positive β-hCG measurement.

DNA fragmentation analysis

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) *in situ* DNA nick end labelling (TUNEL) assay was performed on sperm suspension after density gradient separation as previously described by Benchaib *et al*. (2003). Briefly, part of the sperm sample was used for oocyte insemination, whereas the other part of semen suspension was washed in phosphate-buffered saline (PBS) (Sigma-Aldrich, Milan, Italy), smeared onto microscope slides, fixed in 4% paraformaldehyde (Sigma-Aldrich) in PBS for 30 min at 4°C and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate (Sigma-Aldrich). Strand breaks in DNA were detected by TUNEL using a commercially available kit (*In situ* Cell Death Detection Kit, Fluorescein, Roche, Monza, Italy) according to the manufacturer's instructions. A negative control was performed for each sample by using fluorescein isothiocyanate (FITC)-labelled dUTP without enzyme. The percentage of spermatozoa with fragmented DNA was determined by direct observation of 500 spermatozoa per sample with an epifluorescence microscope (NIKON eclipse 80i, Florence, Italy).

IVF patients were divided in two groups: group A (n = 69), low TUNEL patients (TUNEL positivity <10%); group B (n = 13), high TUNEL patients (TUNEL positivity ≥10%). ICSI patients were also divided in two groups: group C (n = 20), low TUNEL patients (TUNEL positivity <10%); group D (n = 30), high TUNEL patients (TUNEL positivity ≥10%). The threshold value of 10% was chosen in line with the previous study of Benchaib *et al*. (2003) performed using the same technique for sperm preparation (density gradient separation with PureSperm) and for detection of DNA damage on the sperm suspension obtained in this manner (TUNEL assay and evaluation of positive sperms with epifluorescence microscope).

Outcome definitions and statistical analysis

(i) Clinical pregnancy rate was defined as the number of patients with fetal heartbeat divided by the number of treatments;

(ii) Biochemical pregnancy rate was the number of biochemical pregnancies divided by the number of β-HCG positive patients;

(iii) Spontaneous miscarriage rate was the number of spontaneous miscarriages divided by the number of β-HCG positive patients;

(iv) Pregnancy loss rate was the number of biochemical pregnancies and spontaneous miscarriages divided by the number of β-HCG positive patients;

(v) TUNEL positivity rate was the number of patients with DNA fragmentation >10% divided by the number of treatments.

Statistical analysis was performed with SPSS for Windows software package version 10.1 (SPSS, Chicago, IL, USA). The Kolmogorov–Smirnov test was used to determine whether the data were random samples from a normal distribution. The chi-square test or Fisher exact test was used to compare clinical pregnancy rates, pregnancy loss rates and number of previous ART treatments in different groups of patients. *T*-test was applied to analyse maternal age, female BMI and sperm parameters. Spearman's rank correlation, valid for not normally distributed data, was applied to determine the correlation between DNA fragmentation and sperm parameters and between DNA fragmentation and fertilization rate. Statistical differences were considered significant at $P < 0.05$ and highly significant at $P < 0.01$.

Results

In the overall group of ART patients (ICSI + IVF), the clinical pregnancy rate was 22.7%. Biochemical pregnancy and miscarriage rates were 15 and 10%, respectively. Clinical pregnancy rate was 24% in 50 couples undergoing ICSI cycles; biochemical pregnancy and miscarriage rates were 11.8 and 17.6%, respectively. Clinical pregnancy rate was 21.9% in 82 couples undergoing an IVF cycle; biochemical pregnancy and miscarriage rates were 17.4 and 4.3%, respectively.

Patients were divided into groups according to the extent of sperm DNA fragmentation, using a DNA fragmentation threshold value of 10% (Benchaib *et al*., 2003). Of the 132 males examined, 43 (32.6%) were TUNEL positive (DNA fragmentation >10%). Sixty-nine of the 82 males undergoing IVF were TUNEL negative (group A) and 13 were TUNEL positive (group B). Twenty of the 50 ICSI males were TUNEL negative (group C) and 30 were TUNEL positive (group D). Both age and BMI of women of group A and B were not statistically different (age: 37.5 ± 3.75 and 35.61 ± 3.42 years, respectively, $P = 0.095$; BMI: 22.35 ± 3.21 and 23.17 ± 3.64 kg/m^2, respectively, $P = 0.411$); also age and BMI of women in group C and D were not statistically different (age: 36.95 ± 4.91 and 36.72 ± 4.92 years, respectively, $P = 0.872$; BMI: 21.43 ± 2.64 and 21.76 ± 3.12 kg/m^2, respectively, $P = 0.699$). There was no statistical difference in the number of couples who underwent previous ART treatment between groups A and B and between

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groups C and D ($P = 0.356$ and $P = 1.000$, respectively), (Table I). Moreover, there was not a statistical difference between groups A and B and between groups C and D as regards the mean values of sperm parameters (mean values and *P*-values in Table I).

DNA fragmentation and sperm characteristics

Table II summarizes the results of correlation analysis between DNA fragmentation and sperm parameters. Highly significant negative correlations ($P < 0.01$) were observed between sperm DNA fragmentation and all sperm parameters (total number, concentration, motility and morphology), before and after discontinuous gradient centrifugation.

DNA fragmentation and ART outcome

Fertilization rate

A correlation analysis was carried out to determine the relationship between fertilization rate and sperm DNA fragmentation in both ICSI and IVF treatments. The fertilization rate does not appear to have been affected by sperm DNA fragmentation in ICSI ($r = –0.159$; $P = 0.271$), whereas in IVF there was a slight negative correlation between the two parameters ($r = –0.219$; $P = 0.049$).

Clinical pregnancy and pregnancy loss

We evaluated the possible relationship between sperm TUNEL positivity (threshold = 10%) and clinical pregnancy and pregnancy loss in both IVF and ICSI groups (Table I).

As regards the IVF group, clinical pregnancy rates were not statistically significantly different in group A and group B (A versus B = 23.2 versus 15.4%; $P = 0.723$); pregnancy loss rates were also not statistically significantly different in group A and group B (A versus B = 15.8 versus 50%; $P = 0.194$).

In the ICSI group, a highly significant difference was found between group C and group D as regards clinical pregnancy rates (C versus D = 45 versus 10%; $P = 0.007$). There was also a highly significant difference in pregnancy loss between groups C and D, with no biochemical pregnancies or miscarriages found in group C (C versus D = 0 versus 62.5%; $P = 0.009$).

We also tested the possible relationship between DNA fragmentation and clinical pregnancy and pregnancy loss using a 20% threshold, as suggested by other authors (Seli *et al.*, 2004). The results did not significantly change: (i) in the ICSI group, clinical pregnancy and pregnancy loss rates were statistically significantly different in patients with high and low sperm DNA fragmentation ($P = 0.04$ and $P = 0.02$, respectively) and (ii) in IVF group, clinical pregnancy and pregnancy loss rates were not statistically different in the two group of patients ($P = 1.000$).

Table II. Correlation analysis between sperm parameters and sperm DNA fragmentation, before and after density gradient centrifugation

Sperm parameter	Correlation coefficient (*r*)	*P*-value
After density gradient centrifugation		
Sperm concentration	–0.536	<0.001
Total sperm motility	–0.432	<0.001
'Rapid' sperm motility	–0.525	<0.001
Normal sperm morphology	–0.394	<0.001
Before density gradient centrifugation		
Total sperm number	–0.314	<0.001
Sperm concentration	–0.388	<0.001
Total sperm motility	–0.336	<0.001
'Rapid' sperm motility	–0.475	<0.001
Normal sperm morphology	–0.254	0.003

The sperm parameters analysed were total number, concentration, total motility, 'rapid' motility (grade A motility, WHO, 1999) and normal morphology. After density gradient centrifugation and sperm resuspension in 1 ml of culture medium, sperm concentration coincided with total sperm number.

Sperm parameters and ART outcome

Clinical pregnancy and pregnancy loss

We evaluated the relationship between sperm parameters (concentration, motility and morphology), measured on semen before discontinuous gradient centrifugation, and clinical pregnancy and pregnancy loss in both IVF and ICSI groups. In particular, we used WHO threshold levels for normal concentration ($\geq 20 \times 10^6$/ml), motility (progressive motility ≥50%) and morphology (normal forms ≥30%). In both IVF and ICSI groups, there was no statistically significant difference in pregnancy rates between patients with normal or abnormal concentration, motility and morphology. These sperm parameters did not even appear to affect the onset of pregnancy loss: no significant difference in pregnancy loss rates was found between patients with normal or abnormal concentration, motility and morphology (Table III).

Table I. Relationship between TUNEL positivity (low TUNEL <10%, groups A and C; high TUNEL ≥10%, groups B and D) and previous ART treatments, maternal age, female BMI, sperm parameters (before density gradient centrifugation), clinical pregnancy and pregnancy loss rates

	IVF			ICSI		
	A (low TUNEL) ($n = 69$)	B (high TUNEL) ($n = 13$)	*P*-value	C (low TUNEL) ($n = 20$)	D (high TUNEL) ($n = 30$)	*P*-value
Previous ART treatments (%)	39.1	23	0.356	40	36.6	1.000
Maternal age (years)	37.5 ± 3.75	35.61 ± 3.42	0.095	36.95 ± 4.91	36.72 ± 4.92	0.872
Female BMI (kg/m^2)	22.35 ± 3.21	23.17 ± 3.64	0.411	21.43 ± 264	21.76 ± 3.12	0.699
Total sperm number ($\times 10^6$)	174.59 ± 171.17	137.51 ± 83.44	0.449	87.3 ± 98.01	45.48 ± 50.78	0.054
Sperm concentration ($\times 10^6$/ml)	69.77 ± 64.08	37.58 ± 19.06	0.078	34.86 ± 35.43	19.73 ± 26.35	0.090
Total motile sperm (%)	46.45 ± 7.72	42.54 ± 7.46	0.096	40.25 ± 11.97	39.00 ± 13.16	0.735
'Rapid' motile sperm (%)	10.22 ± 5.18	7.31 ± 4.39	0.061	5.75 ± 6.39	3.00 ± 4.28	0.074
Normal sperm morphology (%)	22.9 ± 10.31	19.38 ± 7.97	0.248	15.2 ± 8.29	16.2 ± 6.59	0.638
Clinical pregnancy rate (%)	23.2	15.4	0.723	45	10	**0.007**
Pregnancy loss rate (%)	15.8	50	0.194	0	62.5	**0.009**

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Table III. Fisher exact test results in both IVF and ICSI groups: relationship between concentration ($\times 10^6$/ml), progressive motility, morphology (normal forms) and clinical pregnancies and pregnancy loss rates

	IVF			ICSI		
	Sperm concentration ($\geq 20 \times 10^6$ ml) ($n = 71$)	Sperm concentration ($< 20 \times 10^6$ ml) ($n = 11$)	*P*-value	Sperm concentration ($\geq 20 \times 10^6$ ml) ($n = 19$)	Sperm concentration ($< 20 \times 10^6$ ml) ($n = 31$)	*P*-value
Clinical pregnancy rate	23.9%	9.1%	0.441	26.3%	22.6%	1.000
Pregnancy loss rate	15%	66.7%	0.107	16.6%	36.4%	0.600
	Progressive motility (≥50%) ($n = 11$)	Progressive motility (<50%) ($n = 71$)	*P*-value	Progressive motility (≥50%) ($n = 2$)	Progressive motility (<50%) ($n = 48$)	*P*-value
Clinical pregnancy rate	9.1%	23.9%	0.441	0	25%	1.000
Pregnancy loss rate	0	22.7%	1.000	100%	25%	0.294
	Normal form (≥30%) ($n = 21$)	Normal form (<30%) ($n = 61$)	*P*-value	Normal form (≥30%) ($n = 3$)	Normal form (<30%) ($n = 47$)	*P*-value
Clinical pregnancy rate	19%	23%	1.000	33.3%	23.4%	1.000
Pregnancy loss rate	20%	22.2%	1.000	0	31.3%	1.000

Discussion

Semen quality is usually measured by assessing sperm concentration, motility and morphology (WHO, 1999). These parameters, however, are not able to assess alterations in sperm chromatin organization, such as irregular condensation or DNA damage (Bianchi *et al*., 1996; Sakkas *et al*., 1998). For this reason, sperm DNA fragmentation should be considered during the assessment of semen quality. Various theories have been put forward to explain sperm DNA damage. Some authors think this damage is due to incomplete maturation of the gametes caused by flawed topoisomerase II activity (Bianchi *et al*., 1993; Manicardi *et al*., 1995; Sailer *et al*., 1995); others suggest that the alteration in genetic material is the result of an incomplete apoptotic process (Gorczyca *et al*., 1993; Furuki *et al*., 1996; Rodriguez *et al*., 1997; Sinha Hikim *et al*., 1997; Richburg, 2000), whereas other authors believe that sperm DNA damage may be the result of excess ROS production (Aitken *et al*., 2003). Regardless of sperm DNA damage aetiology, the discovery of cases of male infertility stemming from sperm DNA alteration has raised a new issue. In particular, there is very little information on the possible consequences that fertilization, using sperm with anomalous chromatin organization, may have on embryo development, implantation, gestation and offspring (Sakkas *et al*., 2000a; Seli and Sakkas, 2005). Therefore, simple circumvention of fertilization using ICSI may not overcome all the possible deleterious effects arising from defective sperm DNA (Seli and Sakkas, 2005).

In our study, sperm DNA damage was evaluated by TUNEL assay, performed on part of the sperm suspension prepared by density gradient centrifugation and used in IVF or ICSI. This practice was utilized by several authors (Sun *et al*., 1997; Lopes *et al*., 1998; Duran *et al*., 2002; Benchaib *et al*., 2003; Seli *et al*., 2004). The predictive ability of the sperm DNA integrity test, performed on raw sample, diminishes when spermatozoa are prepared using techniques such as density gradient centrifugation (Sakkas *et al*., 2000b; Tomlinson *et al*., 2001; O'Connell *et al*., 2003; Seli and Sakkas, 2005). The reason why pre-preparation sperm parameters have little prognostic value, in terms of fertilization and pregnancy in ART, may be the 'normalizing' effect of the semen preparation procedure: it is likely that, regardless of the initial sample, a degree of homogenization occurs after sperm preparation. This indicates a need for assessing the sperm DNA status in the appropriate context, that is, sperm DNA damage in raw semen with reference to natural conception and sperm DNA damage in post-preparation samples in relation to ART (Tomlinson *et al*., 2001).

Results of the present study point first to a significant relationship between DNA damage, detected by TUNEL assay in semen samples after density gradient centrifugation, and traditional sperm evaluation parameters. In particular, a highly significant negative correlation was found between degree of DNA fragmentation and total number, concentration, motility and morphology of sperm in both native and treated semen samples (Table II). This result is corroborated by data from other studies (Sun *et al*., 1997; Irvine *et al*., 2000; Benchaib *et al*., 2003). The detection of a close relationship between DNA fragmentation and poor semen quality is reasonable given the aetiology of sperm DNA damage: factors involved in triggering DNA fragmentation (alteration of maturation process, abortive apoptosis or unbalanced ROS production) may also compromise sperm structure and function (Sharma *et al*., 2004). This close relationship is particularly important in ARTs, where the sperm introduced is often selected by an operator (ICSI), the sperm parameters are often 'critical' and, consequently, the risk of injecting sperm with damaged DNA into the oocyte is increased.

Existing data regarding the relationship between sperm DNA integrity and fertilization and pregnancy rates are conflicting (Sun *et al*., 1997; Tomlinson *et al*., 2001; Morris *et al*., 2002; Benchaib *et al*., 2003; Henkel *et al*., 2004; Seli and Sakkas, 2005). Our data show no relationship between sperm DNA damage and fertilization rates in ICSI ($r = -0.159$; $P = 0.271$), whereas in IVF only a slight correlation was found between the two parameters ($r = -0.219$; $P = 0.049$). On the other hand, in ICSI, patients with low DNA fragmentation had a statistically higher clinical pregnancy rate ($P = 0.007$), a relationship that was not found in IVF ($P = 0.723$). These results may be accounted for by the fact that high DNA fragmentation probably does not preclude fertilization but prevents the formation of blastocysts and/or successful embryo development (Ahmadi and Ng, 1999). This is in line with data displayed by Seli and

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colleagues. In their study, the extent of nuclear DNA damage in spermatozoa was related to embryo development to the blastocyst stage, a time when the embryonic genome is activated, transcriptional activity has begun and the paternal genome plays a significant contributory role in embryo function (Seli *et al*., 2004). It is in fact widely assumed that the first steps of development are subjected to maternal control and that the expression of paternal genes begins at 4–8 cell stage; it is therefore at this stage that the consequences of paternal DNA-induced alterations may become manifest, impairing embryo development. In IVF, the lack of any difference in clinical pregnancy rates between high TUNEL patients and low TUNEL patients may be explained by the fact that during IVF there is a kind of 'natural' sperm selection. Sperm that are morphologically anomalous, with poor motility and DNA damage, have low fitness in oocyte fertilization. This idea is partially supported by studies indicating that the zona pellucida may be able to 'identify' genetically altered spermatozoa (Menkveld *et al*., 1991; van Dyk *et al*., 2000).

Our study tried to shed light on any possible relationship between sperm DNA fragmentation and pregnancy loss defined as spontaneous miscarriage or biochemical pregnancy. Our data seem to indicate that, in ICSI, patients with high DNA fragmentation had higher pregnancy loss rates compared to patients with low DNA fragmentation ($P = 0.009$); the same relationship was not found in IVF ($P = 0.194$). These data would seem to substantiate recent studies evaluating the paternal influence on the aetiology of early spontaneous miscarriage (Slama *et al*., 2005), with particular reference to the increased risk of miscarriage because of paternal age and hence sperm chromatin anomalies. Some authors have also carried out DNA fragmentation analyses, using the TUNEL technique, in patients with a history of unexplained recurrent pregnancy loss (RPL) (Carrell and Liu, 2003), showing that many patients with RPL display a significant increase in sperm DNA fragmentation. Parallel studies furthermore show that RPL patients have other genetic alterations such as sperm chromosome aneuploidy (Rubio *et al*., 1999, 2001; Carrell *et al*., 2003). The onset of biochemical pregnancy or early miscarriage may in fact be the result of blocked embryo development because of paternal genomic anomalies. The oocyte is able to trigger repair mechanisms in those cases where it recognizes the presence of sperm damaged DNA. In particular, the oocyte is able to fully repair single-strand DNA breaks. However, if a high level of double-strand DNA breaks are present, the oocyte can produce 'mistakes', generating genetic mutations that can later block or alter embryo development (Braude *et al*., 1988).

Several studies have stressed the importance of traditional sperm parameters as predictors of fertility potential (Nallella *et al*., 2006). Because of the evidence of correlations between sperm DNA fragmentation and clinical pregnancy and pregnancy loss rates in the ICSI group, we also tried to find a relationship between traditional sperm evaluation parameters (concentration, motility and morphology) and pregnancy and pregnancy loss rates in IVF and ICSI groups. In our study, we tried to evaluate the importance of each parameter, individually analysed using current WHO reference values. Our data demonstrate that in both IVF and ICSI groups, sperm quality parameters do not statistically affect clinical pregnancy and pregnancy loss rates (Table III). These results seem to indicate that, despite the strong correlations between traditional sperm parameters and DNA damage and despite the importance of sperm parameters in the evaluation of male infertility (Nallella *et al*., 2006), the analysis of sperm DNA fragmentation has a better predictive value for ART procedure outcome than the use of traditional sperm parameters evaluation. This is in line with others studies indicating that assessment of sperm DNA integrity is more objective and reproducible than analysis of conventional semen parameters (Evenson *et al*., 1991; Zini *et al*., 2001) which are characterized by large within- and between-subject variation (Keel, 2006).

In conclusion, our data indicate that sperm DNA fragmentation significantly affects embryo post-implantation development in ICSI: clinical pregnancy and pregnancy loss rates were significantly different in patients with high and low sperm DNA fragmentation. In particular, we show, for the first time, that sperm DNA fragmentation may compromise progression of pregnancy, resulting in spontaneous miscarriage or biochemical pregnancy following ART. This study also underlines the better predictive value of DNA fragmentation analysis versus traditional sperm parameter evaluation in ART procedure outcome.

Given the importance of sperm DNA integrity, the real cause of DNA damage needs to be assessed with a view to identify proper therapeutic treatment. Furthermore, the development and correct use of methods for selecting sperm with undamaged DNA should be studied carefully, especially where ICSI is strongly recommended.

Acknowledgement

The authors thank Mr Stephen Jewkes for the linguistic revision of the final version of manuscript.

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Submitted on February 23, 2006; resubmitted on April 14, 2006, May 22, 2006; accepted on May 25, 2006

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