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Comparative analysis of three sperm DNA damage assays and sperm nuclear protein content in couples undergoing assisted reproduction treatment

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STUDY QUESTION: Is there an association between sperm DNA damage, measured by three different assays, sperm nuclear protein content and clinical outcomes in assisted reproduction treatment (ART)?

SUMMARY ANSWER: Sperm DNA damage measured by terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling (TUNEL) and the Comet assay were significantly associated with ART outcomes in our single institution study.

WHAT IS KNOWN ALREADY: Abnormal protamine expression is known to be associated with sperm DNA damage and male infertility. A number of studies have shown a significant relationship between sperm DNA damage and ART outcomes. To date, there are no large studies providing direct comparisons of DNA damage tests within the same study population. Thus, the prognostic value for each method remains unknown.

STUDY DESIGN, SIZE, DURATION: Cross-sectional study of 238 men from infertile couples undergoing ART at the University Center for Reproductive Medicine, Utah, USA, between April 2011 and March 2013.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Sperm from men undergoing ART were tested for DNA damage using the alkaline Comet assay, TUNEL and flow cytometric chromatin evaluation (FCCE) assays. Histone retention was analysed using the aniline blue staining method, whereas protamine content (proteins P1 and P2) and ratio were analysed using acid urea gel electrophoresis. The prognostic value of each sperm DNA test to predict clinical pregnancy was calculated.

MAIN RESULTS AND THE ROLE OF CHANCE: Histone retention was associated with sperm DNA damage ($P < 0.001$), reduced embryo quality ($P = 0.005$) and clinical pregnancies ($P < 0.001$). The mean percentage of sperm with DNA damage was significantly higher in sperm from non-pregnant couples compared with that from pregnant couples, as measured by TUNEL assay (15.04 $\pm$ 1.16% versus 8.79 $\pm$ 0.56%; $P < 0.001$) and alkaline Comet assay (72.79 $\pm$ 2.49% versus 55.86 $\pm$ 2.29%; $P < 0.001$). There was no association between clinical pregnancies and DNA fragmentation index measured by FCCE (12.97 $\pm$ 1.46 versus 14.93 $\pm$ 1.65; $P = 0.379$). Of the protamine parameters analysed, only the P1/P2 ratio was associated with sperm count ($P = 0.013$), men's age ($P = 0.037$), maturity ($P = 0.049$) and blastocyst quality ($P = 0.012$). Histone retention and sperm DNA damage measured by Comet and TUNEL assays were associated with fertilization rate ($P < 0.05$), embryo quality ($P < 0.05$) and implantation rate ($P < 0.05$).

LIMITATIONS, REASONS FOR CAUTION: A potential drawback of this study is that it is cross-sectional. Generally in such studies there is more than one variable that could cause the effect. Analysing sperm is one part of the equation; there are also a number of female factors that have the potential to influence ART outcomes. Therefore, given the large and well-established role of female factors in infertility, normal sperm DNA integrity and protamination do not necessarily ensure clinical pregnancy in ART. Thus, female factors can reduce the prognostic value of sperm DNA tests. Further, our use of native semen instead of prepared sperm may have iatrogenically increased the DNA damage.

WIDER IMPLICATIONS OF THE FINDINGS: Alteration in sperm nuclear protein affects sperm DNA integrity. Further, with the current dataset, TUNEL and Comet assays appeared more predictive of ART success than FCCE.

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Introduction

Male infertility impacts 50% of infertile couples. Semen analyses, while helpful, may fail to identify subtle sperm defects that influence assisted reproduction treatment (ART) success, such as condensation defects or DNA strand breaks (Alizadeh *et al.*, 2009). Although morphology is often taken as a surrogate for sperm DNA quality, teratozoospermia has not been shown to impact IVF and ICSI outcomes (Hotaling *et al.*, 2011) and we currently do not have the ability to analyse the genetic potential of an individual spermatozoa without destroying it (Wang *et al.*, 2012). In the past two decades, a broad range of sperm-specific biomarkers have been associated with male infertility and have been described as useful tests to assess sperm function (Aitken, 2006; Breznik *et al.*, 2013) but none of them have had a significant or meaningful impact on clinical management. To date, only sperm nuclear protein and sperm DNA integrity have been shown to have the potential to discriminate between infertile and fertile men (Pacey, 2012).

A number of studies have shown that men with normal semen profiles may still be infertile; abnormal sperm DNA may underlie some of this variation (Host *et al.*, 1999; Saleh *et al.*, 2003; Lee *et al.*, 2010). The sperm genome contributes one half of the offspring's genetic material. Hence, sperm with normal genetic material is essential to obtain a successful pregnancy during natural conception as well as during ART. Considering sperm DNA integrity as a biomarker for male fertility, numerous studies have shown that a high level of sperm DNA damage is associated with an increased time to conception, lower fertilization rates, impaired embryo cleavage, higher miscarriage rates and recurrent pregnancy loss after ART (Evenson *et al.*, 1999, 2007; Spano *et al.*, 2000; Morris *et al.*, 2002; Carrell *et al.*, 2003; Zini *et al.*, 2008; Lewis and Simon, 2010). Unlike somatic cells where DNA is packed in the form of nucleohistone, in sperm 85% of histones are replaced by protamine proteins. Sperm with abnormally low levels of protamine have a higher retention of histone (Aoki *et al.*, 2006a; Alizadeh *et al.*, 2009) which may make the sperm vulnerable to DNA damage (Zhang *et al.*, 2006).

Protamine deficiency and poor protamine packing in sperm is observed in infertile men, suggesting that defective spermatogenesis may lead to alteration in the relative histone-to-protamine ratio in these men (Rosenbusch, 2000; Nasr-Esfahani *et al.*, 2008a,b). In addition, a number of studies have shown a close relationship between abnormal sperm protamination and sperm DNA damage (Rosenbusch, 2000; Nasr-Esfahani *et al.*, 2004, 2005; Torregrosa *et al.*, 2006; Angelopoulou *et al.*, 2007; Plastira *et al.*, 2007; Nili *et al.*, 2009; Tarozzi *et al.*, 2009; Tavalaee *et al.*, 2009; Chiamchanya *et al.*, 2010), supporting the belief that poor chromatin remodelling is a major cause of sperm DNA damage (Bianchi *et al.*, 1993; Zini *et al.*, 2001; Aoki *et al.*, 2006b).

The discrepancies present in the literature suggest that the type of assay used to measure sperm DNA damage may influence the detection of associations with ART outcomes (Huang *et al.*, 2005; Payne *et al.*, 2005; Boe-Hansen *et al.*, 2006; Borini *et al.*, 2006; Muriel *et al.*, 2006; Bakos *et al.*, 2007; Frydman *et al.*, 2008; Lin *et al.*, 2008; Simon *et al.*, 2010, 2011a). Of the three commonly used assays, the alkaline Comet assay and the terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling (TUNEL) assay directly measure the level of DNA damage in sperm, whereas flow cytometric chromatin evaluation (FCCE) using the acridine orange staining method (SCSA® protocol) indirectly measures the susceptibility of DNA to damage. In this study we investigated the correlation between protamine levels and DNA integrity, and associations between the three sperm DNA damage assays and their ability to predict successful pregnancy after ART.

Materials and Methods

Subjects

Men presenting at the University of Utah IVF laboratory, Salt Lake City, UT, USA, for infertility treatment between April 2011 and March 2013 were invited to participate in this study ($n = 238$). All subjects gave written informed consent for participation in this study, and the project was approved by the University of Utah Institutional Review Board. Semen samples were obtained after a recommended 2–5 days of sexual abstinence. All samples were subjected to a conventional light microscopic semen analysis to determine liquefaction, semen volume, sperm concentration, total sperm output and motility according to World Health Organization (WHO) recommendations (World Health Organization, 1999). Following liquefaction, a portion of the sample was used for semen analysis, and at least 100 sperm were analysed. Sperm concentration was determined using a Makler chamber, motility according to WHO guidelines, and morphology following the standard hematoxylin-eosin staining method.

ART procedures

Ovarian stimulation was performed using standard techniques. Oocytes were obtained using ultrasound-guided, transvaginal aspiration. Fertilization was achieved by standard IVF ($n = 89$ cases), ICSI ($n = 149$ cases) or a combination of standard IVF and ICSI ($n = 27$). Standard IVF involved insemination of metaphase II oocytes with 100 000–200 000 progressively motile sperm in a 100 μl drop of human tubal fluid medium. ICSI was performed using microtool sperm immobilization and injection. Resulting embryos were cultured for 3–5 days after oocyte retrieval in human tubal fluid medium, and then transferred to the uterus. All embryos were included in the determination of the patient's mean embryo score (Carrell *et al.*, 1999). Infertility diagnoses for couples included in the study were female factor infertility (40%), male factor infertility (28%) and unexplained factor infertility (32%).

Alkaline Comet assay

Sperm DNA damage was assessed using an alkaline single-cell gel electrophoresis (Comet) assay as modified previously by Hughes *et al.* (1997) and Donnelly *et al.* (1999). Sperm was considered damaged or normal based on the presence or absence of a visible Comet tail, respectively. Fifty to 100 Comets were scored per sample.

TUNEL assay

Assessment of sperm DNA fragmentation was performed using the TUNEL assay as described by Chohan et al. (2006). The assay was performed using the fluorescein *in situ* cell death detection kit following the manufacturer's guidelines (Roche Diagnostics GmbH, Mannheim, Germany). A total of 200 sperm per individual slide was evaluated using fluorescence microscopy by the same examiner.

FCCE assay

The FCCE was performed based on the sperm chromatin structure assay protocol using the acridine orange stain. An aliquot of unprocessed semen (20–100 μl) was diluted to a concentration of $1–2 \times 10^6$ sperm/ml with TNE buffer (0.01 mol/l Tris–HCl, 0.15 mol/l NaCl and 1 mmol/l EDTA, pH7.4). This cell suspension was treated with an acid detergent solution (pH 1.2) 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 purified acridine orange (Polysciences, Inc., Warrington, PA, USA) in a phosphate-citrate buffer, pH 6.0. Cells were analysed using a flow cytometer (Accuri C6, Accuri Cytometers, Inc., Ann Arbor, MI, USA), equipped with an air-cooled argon ion laser.

Aniline blue staining

For assessment of histone retention, unprocessed semen was smeared on a glass slide, air-dried and fixed for 10 min with 4% glutaraldehyde in phosphate-buffered saline. Staining (5 min) was performed using a 5% aniline blue solution that was diluted in 4% acetic acid (approximately pH 3.5). Subsequently, slides were rinsed with water, air-dried and analysed with a bright-field microscope. Overall, 200 sperm were analysed per individual slide. Sperm were classified as previously described (Boitrelle et al., 2011).

Protamine analysis

Purification of nuclear proteins: Sperm nuclear proteins were extracted as previously described from all patients enrolled in the study to determine protamine content and P1/P2 ratios (Carrell and Liu, 2001). *Preparation of the human protamine standard*: A human protamine standard was prepared as previously described (Mengual et al., 2003). The r^2 value of the regression curve was ≥0.96 for each gel run. *Quantification of protamine*: Acetic acid–urea gel electrophoresis was performed as previously described by Carrell and Liu (2001).

Statistical analysis

Data were analysed using the Statistical Package for the Social Sciences (SPSS 18) for Windows (SPSS, Inc., Chicago, IL, USA). The threshold value for sperm DNA damage for the alkaline Comet assay was 18% undamaged or 82% damaged sperm [established in this study based on the odds ratio (OR) value], 10% for TUNEL [previously described by our group (Chohan et al., 2006)] assay and 27% DNA fragmentation index (DFI) for FCCE (Bungum et al., 2004). Our primary outcome was the effect of DNA damage [analysed by Comet ($n = 238$), TUNEL ($n = 235$) and FCCE ($n = 102$)] on clinical pregnancy, evaluated by logistic regression. A clinical pregnancy with fetal heart beat was confirmed by ultrasound on 5 weeks after embryo transfer. The key outcome from the model derived above is individual posterior probabilities of a positive clinical pregnancy. We tested the performance of the prognostic model by calculating the area under the receiver operating characteristic (ROC) curve. Secondary outcomes were fertilization rate, embryo quality (on Days 2, 3 and 5), the percentage of embryos developing to blastocyst and percentage arrested.

The fertilization rate was calculated as the percentage of all fertilized oocytes with two pronuclei for IVF ($n = 89$) and ICSI ($n = 149$). The embryo quality was calculated based on the number of blastomeres and the degree of fragmentation; when a patient had more than one embryo, a mean value was used. Relationships between sperm DNA damage and fertilization and embryo outcomes were compared using the Spearman rank correlation test. Associations between conventional semen parameters and DNA damage were also assessed using the Spearman rank correlation test. To compare the prognostic ability of the different sperm DNA damage tests, we ran logistic regression models with pregnancy (yes/no) as the outcome and with each of the three tests individually as explanatory variables. From these, we estimated thresholds where the predicted probability of a positive pregnancy was equal to 0.1 (ED10). ORs and their 95% confidence intervals (CIs) were computed based on these threshold values. Sensitivities and specificities were calculated above and below the threshold values, together with the ROC and 95% CI for ROC. IVF microdrop and ICSI treatment groups were combined for the analysis.

P1/P2 ratios and protamine content (P1, P2 and P1 + P2) in the native sperm were presented as mean ± SE. Histone retention was expressed as low (no visible stain) and high (>50% stain). Spearman's rank correlation coefficient was used to analyse the relationship between the protamine and histone parameters with semen parameters and sperm DNA damage. The Kruskal–Wallis nonparametric test was used to analyse categories of men's age (≤34, 35–38 and ≥39 years) with the protamine and histone parameters. Analysis of variance with Duncan's *post hoc* test was used to associate categories of fertilization rate (≥70%, good; <70%, low), embryo quality, P1 content (0.15–0.25 μg, normal; <0.15 and >0.25 μg, abnormal), P2 content (0.15–0.25 μg, normal; <0.15 and >0.25 μg, abnormal), total P1 + P2 content (0.30–0.50 μg, normal; <0.30 and >0.50 μg, abnormal), protamine ratio (0.8–1.0, normal; <0.8 and >1.0, abnormal) and histone retention (low and high) with sperm DNA damage (Castillo et al., 2011; Simon and Lewis, 2011).

Results

Comparison of conventional semen profiles from couples that achieved a pregnancy with couples that were unsuccessful following ART

No significant differences between groups were observed in semen volume, total sperm count, normal head morphology and percentage progressive motility. However, couples with unsuccessful pregnancy had a higher sperm concentration than successful couples (mean ± SE: 104.53 ± 7.90 versus 84.87 ± 5.27; $P = 0.033$; Table I).

Correlations between conventional semen parameters and men's age with sperm DNA damage

Sperm DNA damage measured by TUNEL and FCCE assays was associated with semen parameters. However, DNA damage measured by the Comet assay did not correlate with any of the semen parameters (Table II).

Correlations between the three sperm DNA damage measurement assays

The TUNEL assay was associated with the Comet ($r^2 = 0.126$, $P < 0.001$) and FCCE ($r^2 = 0.109$, $P = 0.001$) assays. There was no correlation observed between Comet and FCCE assay ($r^2 = 0.006$, $P = 0.426$).

Table I Demographic data for couples undergoing assisted reproduction treatment (ART).

	Pregnant	Non-pregnant	CI	*P*-value
Cycles included (*n*)[a]	131	104	–	–
Female age (years)	32.69 ± 0.46	34.25 ± 0.54	0.17 to 2.95	0.028
Oocytes retrieved	14.44 ± 0.53	13.67 ± 0.56	−3.30 to 0.76	NS
Oocytes inseminated	11.89 ± 0.48	11.21 ± 0.50	−2.05 to 0.70	NS
Oocytes fertilized (2 pronuclei)	9.44 ± 0.36	8.41 ± 0.43	−2.11 to 0.07	NS
IVF fertilization rate (*n*, %)	44, 75.57 ± 2.45	42, 63.87 ± 2.86	−19.16 to −4.24	0.002
ICSI fertilization rate (*n*, %)	87, 86.07 ± 1.80	61, 85.23 ± 2.12	−6.35 to 4.67	NS
Embryos transferred	2.02 ± 0.04	2.05 ± 0.04	−0.07 to 0.12	NS
Embryo quality (Day 2)	1.23 ± 0.05	1.15 ± 0.05	−0.21 to 0.06	NS
Embryo quality (Day 3)	3.67 ± 0.01	3.39 ± 0.03	−0.61 to 0.06	NS
Blastocyst quality (Day 5)	3.71 ± 0.12	3.56 ± 0.14	−0.22 to 0.51	NS
Blastocyst developed (%)	61.48 ± 1.90	57.05 ± 2.12	−10.15 to 1.30	NS
Blastocyst degenerated (%)	4.24 ± 0.83	7.36 ± 3.12	−2.26 to 8.52	NS
Blastocyst arrested (%)	34.99 ± 1.92	39.07 ± 2.15	−1.71 to 9.88	NS
Men's age (years)	34.44 ± 0.55	35.77 ± 0.59	−0.26 to 2.93	NS
Abstinence time (days)	4.02 ± 0.27	4.08 ± 0.36	−0.95 to 0.84	NS
Semen volume (ml)	3.52 ± 0.15	3.32 ± 0.15	−0.61 to 0.22	NS
Sperm concentration (10^6 ml^{-1})	84.87 ± 5.27	104.53 ± 7.90	1.56 to 37.75	0.033
Total sperm count (10^6)	276.84 ± 18.49	309.77 ± 22.30	−23.68 to 89.54	NS
Progressive motile (%)	23.01 ± 1.17	26.50 ± 1.73	−0.49 to 7.48	NS
Normal head (%)	28.36 ± 1.08	27.94 ± 1.26	−3.67 to 2.84	NS

[a]Embryo transfers were not performed in three patients owing to clinical complications. Values expressed as mean ± SE, CI: confidence interval.

Table II Correlation between semen parameters, men's age and sperm DNA damage.

Semen parameters	Comet assay (*n* = 238)	TUNEL assay (*n* = 235)	FCCE assay (*n* = 102)
Semen volume	$r = 0.019$, P = NS	$r = 0.092$, P = NS	$r = 0.154$, P = NS
Sperm concentration	$r = -0.062$, P = NS	$r = -0.133$, $P = 0.043$	$r = -0.319$, $P = 0.001$
Total sperm output	$r = -0.054$, P = NS	$r = -0.101$, P = NS	$r = -0.231$, $P = 0.019$
Progressive motility	$r = -0.004$, P = NS	$r = -0.159$, $P = 0.015$	$r = -0.490$, $P < 0.001$
Normal morphology	$r = -0.068$, P = NS	$r = -0.211$, $P = 0.002$	$r = -0.290$, $P = 0.004$
Men's age	$r = 0.076$, P = NS	$r = 0.083$, P = NS	$r = 0.128$, P = NS

TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling; FCCE, flow cytometric chromatin evaluation.

Correlations between sperm DNA damage and fertilization rate following ART

There was a decrease in fertilization rate as Comet DNA damage increased in the IVF microdrop insemination group ($r = -0.376$, $P < 0.0001$), but not in the ICSI insemination group ($r = -0.117$, $P = 0.154$). When the fertilization rate was categorized into normal and abnormal DNA damage groups (based on the 82% abnormal Comet threshold value), sperm DNA damage above the threshold value resulted in significantly reduced fertilization rates after IVF and ICSI insemination methods (Fig. 1). There was a decrease in fertilization rates as TUNEL DNA damage increased in the IVF microdrop insemination group ($r = -0.263$, $P = 0.015$), but not in the ICSI insemination group ($r = -0.001$, $P = 0.988$). The results of the FCCE assay did not correlate with fertilization rate.

Correlations between sperm DNA damage and embryo development following ART

Sperm DNA damage measured by the Comet assay was negatively correlated with embryo quality measured on Day 2 ($r = -0.198$, $P = 0.002$), Day 3 ($r = -0.357$, $P < 0.001$), Day 5 ($r = -0.247$, $P = 0.001$) and percentage of blastocysts developed ($r = -0.313$, $P < 0.001$), and was positively correlated with percentage of blastocysts arrested ($r = 0.358$, $P < 0.001$). When the embryo quality was categorized into normal and abnormal DNA damage groups (82% threshold value), there was a reduction in embryo quality in the high DNA damage group on Day 3 and Day 5 (Fig. 2). There was also a significant difference in the percentage of blastocyst development and the percentage of embryos arrested between the groups (Fig. 3). There was no significant correlation between sperm DNA damage measured by the TUNEL and FCCE assays with any of the measured variables.

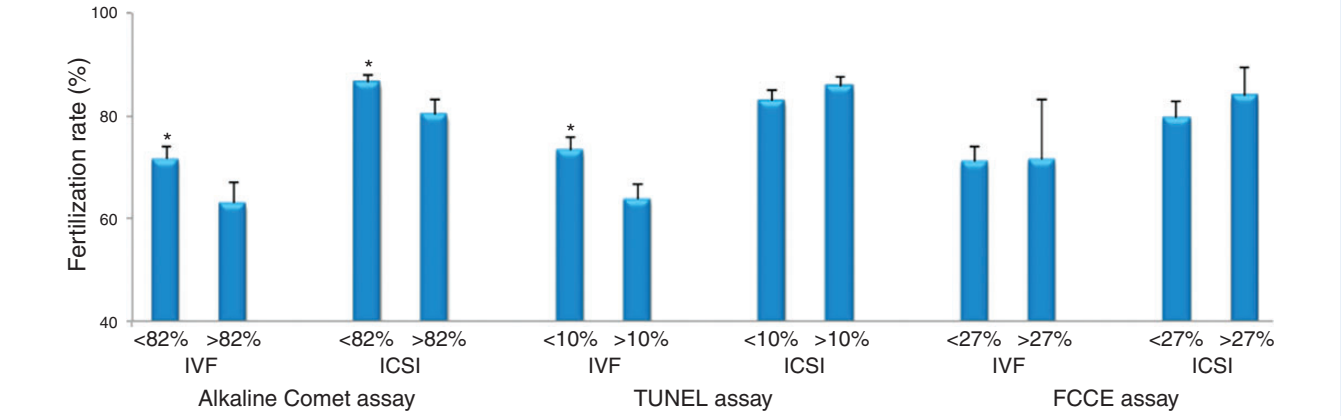

Figure 1 Differences in fertilization rates above and below the threshold value using Comet, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling (TUNEL) and flow cytometric chromatin evaluation (FCCE) assays. Values are mean ± SE, $*P < 0.05$.

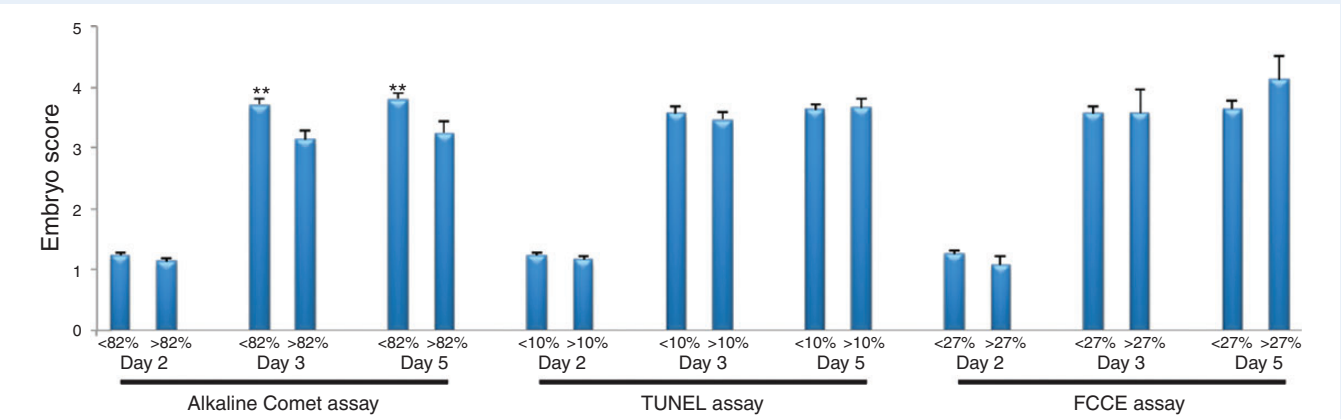

Figure 2 Difference in embryo quality above and below the threshold values using Comet, TUNEL and FCCE assays. $**P < 0.01$.

Correlations between sperm DNA damage and implantation rate following ART

The mean implantation rate was higher when sperm DNA damage was below the threshold value, compared with DNA damage above the threshold value using the Comet (1.04 ± 0.07 versus 0.45 ± 0.09; $P < 0.001$) and TUNEL (1.06 ± 0.08 versus 0.62 ± 0.09; $P < 0.001$) assays. No difference in implantation rate was observed when sperm DNA damage was above and below the threshold value of the FCCE assay (0.83 ± 0.90 versus 0.62 ± 0.77; $P = 0.413$). There was a decrease in sperm DNA damage when more than one embryo was implanted. This relationship was true with Comet and TUNEL assays, but not with FCCE assays (Table III).

Sperm DNA damage of pregnant and non-pregnant couples following ART

Using the Comet and TUNEL assays, the mean percentage of sperm with DNA damage in the native semen was significantly higher in semen from non-pregnant couples ($n = 100$) compared with that from pregnant couples ($n = 129$) undergoing ART (Table IV). However, no such difference was found using the DFI and high density sperm measured by the FCCE assay.

Prognostic value of sperm DNA testing on ART outcomes

Using the threshold values, we calculated OR, ROC curve and relative risk to estimate ART success using the Comet, TUNEL and FCCE assays. The ORs to predict successful pregnancies after ART using the Comet and TUNEL assays were statistically significant, but this was not the case with FCCE assay (Fig. 4; Table V).

Correlations between semen parameters and sperm DNA damage with histone retention

Histone retention was associated with semen volume and normal sperm morphology but not with sperm count and sperm motility. Histone retention was associated with sperm DNA damage measured by

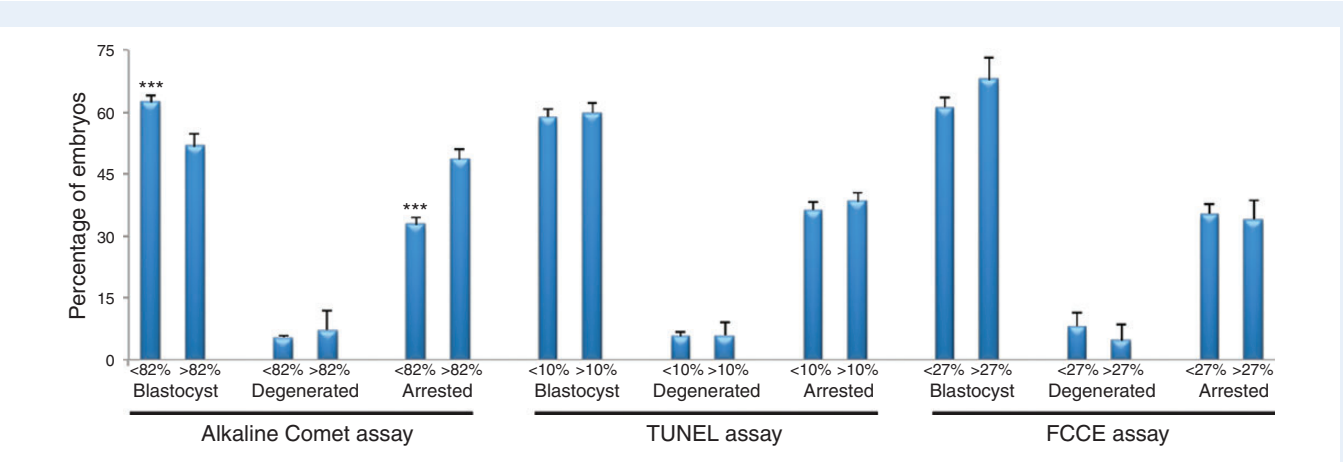

Figure 3 Difference in blastocyst development, embryos degenerated and embryo arrest above and below the threshold value using Comet, TUNEL and FCCE assays. ***$P < 0.001$.

Table III Comparison between sperm DNA damage and number of embryos implanted following embryo transfer.

Implantation	Comet assay	TUNEL assay	FCCE assay
No implantation			
n (%)	100 (43.7)	100 (44.3)	46 (47.9)
DNA damage	72.79 ± 2.48[a]	15.23 ± 1.14[a]	12.97 ± 1.45
1 embryo			
n (%)	61 (26.6)	60 (27.4)	25 (24.0)
DNA damage	59.92 ± 2.96[b]	8.98 ± 0.83[b]	13.85 ± 2.83
2 embryo			
n, (%)	68 (28.4)	66 (27.0)	25 (26.0)
DNA damage	51.34 ± 3.38[b,c]	8.72 ± 0.76[b,c]	14.02 ± 1.75

Values with different superscripts within each column are significantly different ($P < 0.05$).
The values of DNA damage are expressed as mean ± SE.

Comet, TUNEL and FCCE assays (Table VI). There was no association between histone retention and men's age.

Correlations between histone retention and ART outcomes

There was a significant correlation between histone retention and early embryo development on Day 2 and Day 3. An abnormally high level of histone retention was negatively associated with embryo quality on Day 2 ($r = -0.190$, $P = 0.009$) and Day 3 ($r = -0.206$, $P = 0.005$). Absence or low levels of histone retention were positively associated with Day 2 ($r = 0.203$, $P = 0.005$) and Day 3 ($r = 0.204$, $P = 0.005$) embryo development. However, histone retention was not associated with fertilization rate or blastocyst quality. Couples achieving clinical pregnancy had an increased percentage of sperm with normal histone and a decreased percentage of sperm with abnormal histone retention compared with couples with failed pregnancies (Fig. 5).

Correlation between the protamine content and P1/P2 ratios with sperm DNA damage

A significant negative correlation between sperm DNA damage and protamine content was observed with protamine 1 ($r = -0.247$, $P = 0.009$), protamine 2 ($r = -0.231$, $P = 0.015$) and total P1 + P2 protamines ($r = -0.242$, $P = 0.011$) based on the Comet assay. No significant correlations were observed between sperm DNA damage with protamine content based on TUNEL and FCCE assays. The P1 and P2 content was lower in men with sperm DNA damage above the threshold value. However, no such correlations were observed with protamine ratio (Table VII; Fig. 6).

Correlation between protamine content and P1/P2 ratios with semen parameters, histone retention and ART outcomes

Of the classic semen parameters, sperm motility was negatively associated with P1 content ($r^2 = -0.188$, $P = 0.05$), and P1/P2 ratio was negatively associated with semen volume ($r^2 = -0.257$, $P = 0.007$) and sperm count ($r^2 = -0.237$, $P = 0.013$). A negative association was observed between men's age and P1/P2 ratio ($r^2 = -0.211$, $P = 0.037$). There was a positive correlation between normal histone retention and total (P1 + P2) protamine content ($r^2 = 0.189$; $P = 0.049$). Protamine 2 level was positively associated with percentage of sperm with normal histone retention ($r^2 = 0.188$; $P = 0.050$). Protamine 2 content was positively associated with the percentage of blastocysts developed ($r^2 = 0.207$; $P = 0.050$). When protamine content and ratio were categorized into normal and abnormal categories, none of the protamine parameters were associated with ART outcomes.

Discussion

This study associates the effect of sperm DNA damage measured by the Comet, TUNEL and FCCE assays and sperm nuclear protein content with ART outcomes in a cohort of 238 couples. Abnormalities in the sperm nuclear proteins, such as histones and protamines, were associated with sperm DNA damage, while a weak association was observed

Table IV Comparison of DNA damage between pregnant and non-pregnant couples after ART.

DNA test	Pregnant couples (n = 129)	Non-pregnant couples (n = 100)	Difference (95% CI)	P-value	ROC
Comet	55.40 ± 2.29	72.79 ± 2.49	17.39 (−24.09, −10.70)	<0.001	0.648
TUNEL	8.71 ± 0.56	15.23 ± 1.15	6.52 (−8.88, −4.16)	<0.001	0.629
FCCE	14.93 ± 1.65	12.97 ± 1.46	−1.96 (−2.45, 6.37)	0.379	0.440

The values are expressed as mean ± SE.
ROC, receiver operating characteristic curve.

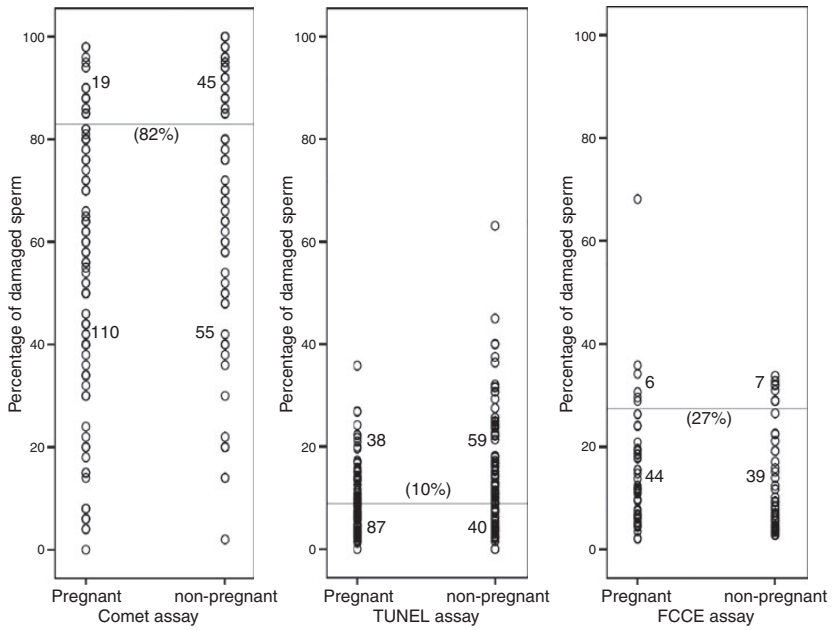

Figure 4 DNA damage distribution measured by Comet, TUNEL and FCCE assays according to pregnancy outcome. The threshold values for each assay are represented by a line. The n values above and below the threshold values are provided.

Table V Prognostic value of sperm DNA damage in predicting clinical pregnancy after ART.

	Comet assay	TUNEL assay	FCCE assay
Threshold value (%)	82	10	27
Odds ratio (95% CI)	7.00 (3.62, 13.94)	3.38 (1.87, 6.11)	1.32 (0.36, 4.91)
Sensitivity (%)	85.27	69.60	88.00
Specificity (%)	45.00	59.60	15.22
PPV (%)	66.67	68.50	52.08
NPV (%)	70.31	60.82	53.85
Relative risk (95% CI)	1.89 (1.51, 2.38)	1.72 (1.32, 2.25)	1.04 (0.88, 1.22)

PPV, positive predictive value; NPV, negative predictive value.

between nuclear proteins and ART outcomes. We noted a strong relationship between sperm DNA damage and ART outcomes (fertilization rate, embryo quality, blastocyst quality, implantation rate and pregnancy rate). The predictive power was significantly higher using the alkaline Comet assay when compared with TUNEL and FCCE assays. There was a significant decrease in pregnancy rates in patients when the percentage of damaged sperm was above the threshold value of 82% measured by Comet assay and 10% threshold value measured by TUNEL assay.

Table VI Correlations between semen parameters and DNA damage with histone retention.

Parameters	No histone retention	Abnormal histone retention
Semen volume	$r = -0.162$, $P = 0.027$	$r = 0.166$, $P = 0.022$
Sperm concentration	$r = 0.110$, $P =$ NS	$r = -0.090$, $P =$ NS
Total sperm output	$r = 0.060$, $P =$ NS	$r = -0.038$, $P =$ NS
Progressive motility	$r = 0.066$, $P =$ NS	$r = -0.059$, $P =$ NS
Normal morphology	$r = 0.149$, $P = 0.045$	$r = -0.145$, $P = 0.050$
DNA damage – Comet	$r = -0.329$, $P < 0.001$	$r = 0.331$, $P < 0.001$
DNA damage – TUNEL	$r = -0.413$, $P < 0.001$	$r = 0.424$, $P < 0.001$
DNA damage – FCCE	$r = -0.214$, $P = 0.032$	$r = 0.203$, $P = 0.041$
Men's age	$r = -0.053$, $P =$ NS	$r = 0.046$, $P =$ NS

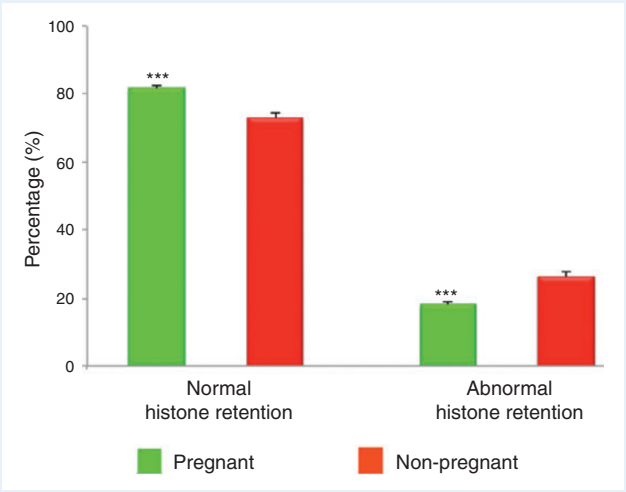

Figure 5 Bar chart showing difference in the percentage of sperm with histone retention between pregnant and non-pregnant couples following ART. $P < 0.001$.

A strong negative relationship was observed between sperm DNA damage analysed by TUNEL and FCCE assays and sperm concentration, progressive motility and normal morphology. The current literature shows mixed data, with some studies showing good association and others finding no correlations (Greco *et al.*, 2005; Nicopoullos *et al.*, 2005; Zini *et al.*, 2005; Ozmen *et al.*, 2007; Lin *et al.*, 2008; Nijs *et al.*, 2009; Tavalaee *et al.*, 2009). Such controversies may arise due to variability in laboratory conditions, lack of standard protocols for DNA damage assays and inter- and intra-individual variability in the test parameters. In the current study 59% of ART patients showed normal semen profiles according to the WHO criteria. Of these patients 24% had DNA damage above the threshold value for the Comet assay ($P = 0.105$), 40% had DNA damage above the threshold value for the TUNEL assay ($P = 0.419$) and 9% had DNA damage above the threshold value for the FCCE assay ($P = 0.225$).

Numerous techniques have been used to study sperm DNA abnormalities. These methods can be broadly classified into direct and indirect methods of assessment. In the Comet assay, DNA damage is quantified by measuring the displacement of broken DNA strands between the comet head and tail when subjected to electrophoresis (Singh *et al.*, 1988). A unique and powerful feature of the Comet assay is that it can detect variable levels of DNA damage within individual cells and it is the only test that can show 100% of the sperm population being damaged (Ribas-Maynou *et al.*, 2013). We preferred the alkaline Comet assay to the neutral Comet assay as the latter measures only double-strand breaks, whereas the alkaline version measures both single- and double-strand breaks. Earlier studies (Simon *et al.*, 2010, 2011a,b, 2012) have established threshold values by calculating the mean of DNA strand breaks in 50 sperm. In this study the percentage of damaged sperm was analysed for the first time to estimate a threshold value 82% of sperm with Comet DNA damage. This threshold value was established based on the ART outcome (clinical pregnancy). A damaged sperm is characterized based on the presence of a Comet tail. The threshold value of 82% of sperm with DNA damage is comparable to the mean value of 56% Comet DNA damage published previously (Simon *et al.*, 2010). Previously, other quantitative parameters, such as tail length and olive tail moment, have also been used to assess the level of DNA damage in sperm (Morris *et al.*, 2002; Tomsu *et al.*, 2002).

The sensitivity of the alkaline Comet assay to identify a damaged sperm is very high compared with other assays. In this study, a threshold value of 82% for the Comet assay was established, significantly higher than the thresholds of 10% for TUNEL and 27% DFI for FCCE (Fig. 4). Such variations in threshold values can be explained by analysing the mean, median and range for each assay. The alkaline Comet assay yielded a mean (63.9%), median (66%) and range (0–100%) of sperm with DNA damage, whereas for the TUNEL assay the values were 11.4, 8.9 and 0–63%, and for the FCCE the values were 13.8, 9.9 and 0–66%, respectively. The alkaline Comet assay is the only sperm DNA damage assessment test so far to show a distribution of damaged sperm populations ranging from 0 to 100% (Ribas-Maynou *et al.*, 2013). Despite a high threshold value of 82% damaged sperm, 28% of the patients analysed had DNA damage above the threshold value. In this study, we used a 10% threshold value for the TUNEL assay based on a previous study in our laboratory (Chohan *et al.*, 2006), which identified the point at which the highest odds value to determine a clinical pregnancy was obtained.

Sperm DNA damage measured by the TUNEL assay resulted in a strong positive correlation with the Comet as well as FCCE assay. Similar observations were previously reported by Zini *et al.* (2001), Erenpreiss *et al.* (2004) and Chohan *et al.* (2006). A strong correlation

Table VII Effect of protamine (P1 and P2 proteins) parameters on sperm DNA damage. The values are expressed as percentage.

Assay	Threshold value	(*n*)	P1/P2 ratio			P1 (ng)		P2 (ng)	
			<0.8	0.8–1.0	>1.0	<200	>200	<200	>200
Comet	<82%	90	28	59	13	54	46	37	63
	>82%	22	23	50	27	73	27	55	45
TUNEL	<10%	72	22	60	18	57	43	35	65
	>10%	40	35	53	12	65	35	50	50
FCCE	<27%	74	18	66	16	55	45	34	66
	>27%	10	10	50	40	70	30	60	40

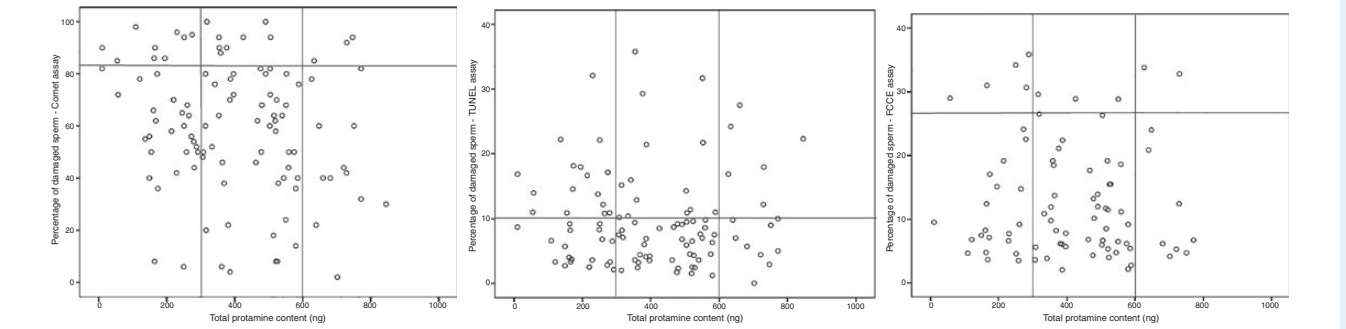

Figure 6 Correlation between DNA damage (% of damaged sperm) and total protamine content (P1 + P2). Vertical lines represent abnormally low and abnormally high levels of protamine. Horizontal lines represent the threshold value for each DNA damage assay.

between two parameters does not necessarily mean that both parameters are the same (Henkel *et al.*, 2010). It is merely an indication that the two parameters are somehow related, and such a relationship can even be indirect. On the other hand, in this study the association between DFI measured by FCCE and the Comet assay is not only markedly weaker but also much less significant than has been previously reported. The difference between FCCE and the Comet assay is that the FCCE detects 'potential' DNA damage and the susceptibility to denaturation, while the Comet assay detects complete DNA damage. During the Comet protocol, the sperm is lysed and the nucleus is completely decondensed exposing the strand breaks present in the nuclear core. This concept also explains why thresholds for the Comet assay are normally higher than those for the FCCE and TUNEL assays and why the Comet assay may have higher predictive values than the other assays that only detect the potential for DNA damage.

This study shows that histone retention is positively associated with sperm DNA damage. Ward (2010) proposed a unique hypothesis on the structural arrangement of histone- and protamine-bound regions of DNA in the mature sperm where protamine-bound DNA is protected from damage by the toroidal compaction and subsequent stacking of toroids, while the histone-bound regions of DNA are vulnerable to degradation by endonucleases and oxidative stress mediated damage (Dominguez *et al.*, 2011). This structural packaging may help to explain our results and those of other studies showing a positive correlation between histone retention and sperm DNA damage (Aoki *et al.*, 2005b; Garcia-Peiro *et al.*, 2011). In support of this hypothesis we also observed a significant correlation between histone retention and total protamine and P2 content.

A number of studies have shown an association between the quality of chromatin packing in sperm and fertility status of men, and the deteriorating effect of abnormal chromatin packing on ART outcomes (Nasr-Esfahani *et al.*, 2001; Esterhuizen *et al.*, 2002; Razavi *et al.*, 2003). In support of this, we observed a significant increase in sperm DNA damage with a reduction in protamine content. In addition, the P1 and P2 content was lower in men with sperm DNA damage above the threshold values. Protamines facilitate strong intermolecular attraction between the positively charged protamine and the negatively charged DNA to yield a compact DNA–protamine structure (Aoki and Carrell, 2003). A close association between sperm DNA integrity and protamine suggests that the damage may be due to a defective spermiogenesis process (Evenson *et al.*, 2000). Previously it has been shown that protamines play a major role in protecting sperm chromatin from damage, and abnormalities in protamine content may render the sperm DNA vulnerable to damage (Aoki *et al.*, 2006b).

The present study evaluates the relationship between sperm nuclear proteins and ART outcomes. Embryo quality appeared to be affected by P2 content, whereas fertilization and clinical pregnancy were not affected in patients with abnormal protamine content or ratio. We did not observe any significant association between normal and abnormal categories of protamine content or ratio with ART outcomes. These

results are consistent with studies published earlier by our group and others showing fewer associations between abnormal protamine expression and ART outcomes (Carrell and Liu, 2001; Aoki and Carrell, 2003; Razavi *et al.*, 2003; Aoki *et al.*, 2005a). It does not appear as if normal protamination is required for normal fertilization, because ICSI with round spermatids has been shown to produce chromatin decondensation and pronucleus formation (Ahmadi and Ng, 1999). In contrast to these results, some studies have concluded that alteration in protamine content can affect fertilization and embryo quality, which subsequently may affect implantation and pregnancy outcome (Nasr-Esfahani *et al.*, 2004).

In contrast to protamines, histone retention analysed by the aniline blue assay showed an adverse effect on embryo development and clinical pregnancy. Our results are consistent with those of Hammadeh *et al.* (1998) showing that chromatin condensation visualized by aniline blue staining is a good predictor of ART outcomes. We also observed a strong positive correlation between histone staining and sperm DNA damage analysed by the Comet, TUNEL and FCCE assays and a negative correlation between histone retention and protamine content. We hypothesize that abnormal histone retention may result in reduced chromatin condensation. Subsequently, the DNA nicks are normally repaired by this same enzyme prior to completion of spermiogenesis (Laberge and Boissonneault, 2005). If the replacement of histones by protamines is incomplete, the nicks may not be repaired resulting in sperm with DNA damage (Muratori *et al.*, 2006).

Although there has been a significant amount of research associating sperm DNA damage with ART outcomes, the results are controversial (Micinski *et al.*, 2009; Nijs *et al.*, 2009, 2011; Tavalaee *et al.*, 2009; Avendano *et al.*, 2010; Speyer *et al.*, 2010; Kennedy *et al.*, 2011; Meseguer *et al.*, 2011; Simon *et al.*, 2011a; Breznik *et al.*, 2013; Dar *et al.*, 2013). The meta-analysis of Zini and Sigman (2009) and of Collins *et al.* (2008) found a weak association of sperm DNA damage against ART outcomes. Whereas the meta-analysis of Li *et al.* (2006) comparing sperm DNA damage measured by TUNEL and FCCE indicated that clinical pregnancy rates decreased significantly for patients with high sperm DNA damage as assessed by TUNEL assay, no such correlations were observed with FCCE. Such conflicts in the literature are probably due to differences in methodology, and our understanding of the effect of sperm DNA damage on reproductive outcomes is far from complete. To date all DNA damage tests are assumed to provide the same value in correlating sperm DNA damage with ART outcomes (Collins *et al.*, 2008; Zini and Sigman, 2009). However, the currently available sperm DNA tests measure different aspects of DNA damage and have variable levels of prognostic value (Lewis, 2013). Therefore, a large cross-sectional study comparing all the methods on the same population is necessary to reach a more consistent conclusion.

In this study, we analysed sperm DNA damage using three methods (Comet, TUNEL and FCCE—SCSA® protocol) in men undergoing ART and we associated the level of sperm DNA damage with their clinical outcomes. We observed a negative correlation between sperm DNA damage and fertilization rate with the Comet and TUNEL assays. In addition, a significant decrease in the fertilization rate was observed when sperm DNA damage was above the threshold value using Comet and TUNEL assays. Such correlations were observed following the IVF insemination method but not after ICSI treatment. However, no significant correlations were observed with DNA damage analysed by the FCCE assay, either after IVF or ICSI. This is in agreement with numerous studies showing a marked negative correlation between sperm DNA damage and fertilization rate after IVF based on the Comet assay (Simon *et al.*, 2010, 2011a,b, 2012; Simon and Lewis, 2011) and TUNEL assay (Huang *et al.*, 2005; Payne *et al.*, 2005; Borini *et al.*, 2006; Bakos *et al.*, 2007; Breznik *et al.*, 2013). In contrast, several studies have found no correlation between sperm DNA damage and IVF fertilization rate (Benchaib *et al.*, 2007; Frydman *et al.*, 2008; Esbert *et al.*, 2011). However the results do agree with those from Borini *et al.* (2006) and Breznik *et al.* (2013) showing that sperm DNA damage negatively affects fertilization rates following IVF but not ICSI treatment. The adverse effects of DNA damage seen here may be due to abnormal chromatin packing in sperm which is associated with high DNA damage (Simon *et al.*, 2011b) and also with a failure of sperm DNA to decondense post-fertilization (Sakkas *et al.*, 1996; Lopes *et al.*, 1998).

Our study also showed a significant correlation between sperm DNA damage and embryo quality, and this relationship was true with the Comet assay. In addition, a significant decrease in embryo quality was observed when sperm DNA damage was above the threshold value of the Comet assay. These results are in disagreement with the findings of Braude *et al.* (1988) who showed that DNA damage in sperm is not important in early embryogenesis, and that until the 4–8 cell embryonic stage the oocyte genome controls early development. Only after this stage does the embryonic genome become transcriptionally active with the paternal genome contributing to further embryo development. In contrast, our study shows that sperm DNA damage adversely impacts embryo quality as early as Day 2, while the negative impact of sperm DNA damage is even more pronounced during the blastocyst stage.

We report that sperm DNA damage assessed by TUNEL and FCCE assays was not associated with embryo or blastocyst quality. Our results are consistent with other studies showing no significant association between embryo development and sperm DNA damage assessed by TUNEL (Bakos *et al.*, 2007; Frydman *et al.*, 2008; Esbert *et al.*, 2011) and SCSA (Payne *et al.*, 2005; Bungum *et al.*, 2007; Lin *et al.*, 2008; Micinski *et al.*, 2009; Speyer *et al.*, 2010). In contrast, a few studies have reported a significant association between embryo development and sperm DNA damage (Saleh *et al.*, 2003; Seli *et al.*, 2004; Virro *et al.*, 2004; Benchaib *et al.*, 2007). These discrepancies are probably due to the type of DNA damage assays used, as these assays measure different aspects of DNA damage. Secondly, the sensitivity of TUNEL and FCCE assays to detect DNA damage are low compared with Comet assay (Ribas-Maynou *et al.*, 2013) which may be attributed to the fact that decondensation of the sperm nucleus is necessary to measure the total DNA damage (de Iuliis *et al.*, 2009). The threshold value for the Comet assay (82%) is significantly higher than those of TUNEL (10%) and FCCE (27%) assays owing to the sensitivity of the Comet assay where all double-strand and single-strand breaks are revealed after complete decondensation while for the other assays only peripheral DNA damage is accessible.

This study shows that sperm DNA damage correlates with implantation efficiency. Our results are consistent with those of studies showing a high implantation rate in patients when sperm DNA damage is below the threshold value (Frydman *et al.*, 2008). Kennedy *et al.* (2011) was the first study to examine the association between sperm DNA damage and multiple pregnancy rates, and reported significantly lower sperm DNA damage in men from couples having triplet pregnancies compared with those who had a spontaneous abortion. To date, few

studies have associated the relationship between sperm DNA damage and the incidence of multiple implantation or multiple births in couples undergoing ART. This is consistent with our earlier results in which we found that sperm DNA damage affected early embryo development, and in patients with low sperm DNA damage >50% of the embryos developed into blastocysts. These results indicate that sperm DNA damage may affect implantation rates and be associated with rates of spontaneous pregnancy loss (Benchaib *et al.*, 2007; Ozmen *et al.*, 2007; Frydman *et al.*, 2008; Lin *et al.*, 2008), a phenomenon termed 'late paternal effect' by Tesarik *et al.* (2004). Therefore, we could speculate that decision-making in ART could be adjusted based on sperm DNA damage to reduce the chances of multiple births.

In this study, couples that achieved a clinical pregnancy following ART had significantly lower sperm DNA damage than couples that did not. The association was significant when sperm DNA damage was assessed by Comet and TUNEL assays, but not with FCCE. Our results on clinical pregnancies were consistent with previous reports using the Comet assay (Simon *et al.*, 2010, 2011a,b, 2012), whereas using the TUNEL and FCCE assays, the literature remains unclear on the impact of sperm DNA damage on clinical pregnancies after ART (Huang *et al.*, 2005; Payne *et al.*, 2005; Boe-Hansen *et al.*, 2006; Borini *et al.*, 2006; Bakos *et al.*, 2007; Benchaib *et al.*, 2007; Bungum *et al.*, 2008; Frydman *et al.*, 2008; Lin *et al.*, 2008; Micinski *et al.*, 2009; Nijs *et al.*, 2009, 2011; Avendano *et al.*, 2010; Esbert *et al.*, 2011). An explanation for the conflicts in the reported literature may be due to patient inclusion factors. Most of the studies have included couples with female factors; therefore the effect of sperm DNA damage on pregnancy outcome is compromised by female infertility factors (Simon *et al.*, 2011a). For example, in the studies Payne *et al.* (2005), Frydman *et al.* (2008), Nijs *et al.* (2009) and Meseguer *et al.* (2011) more than half of the couples had been diagnosed with female infertility. The inclusion of patients with female infertile factor could reduce the prognostic value of the sperm DNA tests (Giwercman *et al.*, 2009).

To assess the clinical usefulness of the sperm DNA tests, their threshold value and ORs must be calculated. Previous studies on the Comet assay have reported a threshold value of 56% (Simon *et al.*, 2010) and 52% (Simon *et al.*, 2011a) by calculating the mean across the DNA damage values for each sperm. However, in this study for the first time a threshold value for Comet assay was calculated by assessing the percentage of sperm with DNA damage. We report a threshold value of 82% of sperm with DNA damage, which was calculated across 238 subjects. This value also corresponds to the highest OR to obtain a successful clinical pregnancy. A 10% threshold value was calculated for the TUNEL assay, which was previously reported by Borini *et al.* (2006) and Ozmen *et al.* (2007).

The ORs to predict successful pregnancies after ART were 7.00 for Comet, 3.38 for TUNEL and 1.32 for FCCE. This OR for Comet is higher than the overall ORs obtained by the meta-analysis when all the studies were combined (Collins *et al.*, 2008; Zini and Sigman, 2009), suggesting an improved efficiency of the alkaline Comet assay compared with the SCSA and TUNEL assays which were included in the meta-analysis. Interestingly, the TUNEL assay resulted in a lower OR but did show a statistically significant difference in pregnancy outcome. This may be due to the fact that both Comet and TUNEL assays measure the direct level of DNA damage (Martins *et al.*, 2007), whereas SCSA measures the susceptibility of the sperm DNA to damage (Evenson and Jost, 2000).

When the three assays were compared to identify the tests with high prognostic value to predict clinical pregnancies, Comet and FCCE assays showed higher sensitivity but the specificity was higher using Comet and TUNEL assays. The results of the OR was also confirmed by the ROC curve where Comet (0.648) and TUNEL (0.629) assays showed the area under the curve to differentiate couples that had a successful pregnancy from those who did not. The DNA damage assays had a low positive predictive value (52–68.5%). The false positives may be related to the presence of female infertility factors in the couples undergoing ART treatment. Approximately 40% of the couples enrolled in this study showed the presence of female infertility issues which could have prevented a positive pregnancy despite a low level of sperm DNA damage. Men with sperm DNA damage above the threshold values had an increased relative risk of not achieving a clinical pregnancy for Comet (1.89) and TUNEL (1.72) assays, compared with the FCCE assay (1.04). In addition, the positive and negative predictive values of Comet and TUNEL assays were higher compared with FCCE assay, once again indicating that these two tests have higher prognostic value than the FCCE assay. Our results are in agreement with previously reported studies showing that the DFI does not provide sufficient information about the complete extent of DNA damage in sperm (Frazer, 2005), which may explain its low prognostic value. Another explanation for this effect may be the sample size in our study, where the Comet and TUNEL assays were used in more samples than the FCCE assay.

In conclusion, abnormalities in histone and protamine content were associated with increased sperm DNA damage. The level of sperm DNA damage measured by the Comet assay was comparable to the TUNEL assay but not to FCCE. In the Comet assay, the method of analysing the percentage of sperm with DNA damage is more effective when compared with the mean level of DNA damage across each sperm. The sensitivity and specificity of the alkaline Comet assay in predicting ART outcome is higher compared with TUNEL and FCCE assays. This study provides robust evidence that sperm DNA damage as a useful biomarker in helping to predict ART outcome, and assessment of DNA damage in sperm using the alkaline Comet and TUNEL assays provide the sensitivity and specificity needed to best predict ART success. The development of novel diagnostic approaches that allow non-invasive evaluation of individual sperm to select sperm with minimal DNA damage could be a potentially valuable tool to improve ART success.

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Authors' roles

L.S. is a postdoctoral fellow and was responsible for performing sperm DNA damage assays, statistical analysis and writing the manuscript. L.L. and S.G. performed the protamine quantification and aniline blue assays. K.M. was responsible for collecting ART data from patients enrolled in the study. J.H. and K.I.A. contributed to the writing of the manuscript and analysis of results. B.E. was responsible for consenting the study subjects. D.T.C. is the principle investigator for this study and is responsible for design of the study. D.T.C. is the head of ART unit and is the corresponding author for this manuscript.

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Conflict of interest

D.T.C. has received no personal financial support for this work. L.S., L.L., K.M., S.G., J.H., K.I.A. and B.E. have no conflict of interest.

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