Clinical significance of sperm DNA damage in assisted reproduction outcome

Simon, L., Stevenson, M., Lutton, D., McManus, J., Lewis, S., & Brunborg, G. (2010). Clinical significance of sperm DNA damage in assisted reproduction outcome. *Human reproduction*, *25*(7), 1594-1608. https://doi.org/10.1093/humrep/deq103

Published in:
Human reproduction

Human Reproduction, Vol.25, No.7 pp. 1594–1608, 2010
Advanced Access publication on May 6, 2010 doi:10.1093/humrep/deq103

human reproduction **ORIGINAL ARTICLE** ***Andrology***

Clinical significance of sperm DNA damage in assisted reproduction outcome

Luke Simon [1], Gunnar Brunborg [2], Michael Stevenson [3], Deborah Lutton [4], Joanne McManus [4], and Sheena E.M. Lewis [1,*]

[1]Centre for Public Health, Reproductive Medicine, Institute of Clinical Science, Queens University of Belfast, Grosvenor Road, Belfast BT12 6BJ, UK [2]Norwegian Institute of Public Health, PO Box 4404, Nydalen, Oslo N-0403, Norway [3]Centre for Public Health, Mulhouse Building, Queens University of Belfast, Grosvenor Road, Belfast BT12 6BJ, UK [4]Regional Fertility Centre, Royal Jubilee Maternity Service, Grosvenor Road, Belfast BT12 6BA, UK

*Correspondence address. Tel: +44-28-9063-3987; Fax: +44-28-9032-8247; E-mail: s.e.lewis@qub.ac.uk

Submitted on January 27, 2010; resubmitted on March 3, 2010; accepted on March 29, 2010

BACKGROUND: Sperm DNA damage shows great promise as a biomarker of infertility. The study aim is to determine the usefulness of DNA fragmentation (DF), including modified bases (MB), to predict assisted reproduction treatment (ART) outcomes.

METHODS: DF in 360 couples (230 IVF and 130 ICSI) was measured by the alkaline Comet assay in semen and in sperm following density gradient centrifugation (DGC) and compared with fertilization rate (FR), embryo cumulative scores (ECS_1) for the total number of embryos/treatment, embryos transferred (ECS_2), clinical pregnancy (CP) and spontaneous pregnancy loss. MB were also measured using formamidopyrimidine DNA glycosylase to convert them into strand breaks.

RESULTS: In IVF, FR and ECS decreased as DF increased in both semen and DGC sperm, and couples who failed to achieve a CP had higher DF than successful couples (+12.2% semen, $P = 0.004$; +9.9% DGC sperm, $P = 0.010$). When MB were added to existing strand breaks, total DF was markedly higher (+17.1% semen, $P = 0.009$ and +13.8% DGC sperm, $P = 0.045$). DF was not associated with FR, ECS or CP in either semen or DGC sperm following ISCI. In contrast, by including MB, there was significantly more DNA damage (+16.8% semen, $P = 0.008$ and +15.5% DGC sperm, $P = 0.024$) in the group who did not achieve CP.

CONCLUSIONS: DF can predict ART outcome for IVF. Converting MB into further DNA strand breaks increased the test sensitivity, giving negative correlations between DF and CP for ICSI as well as IVF.

Key words: Comet assay / formamidopyrimidine DNA glycosylase enzyme / modified base / sperm DNA fragmentation / threshold value

Introduction

Infertility is becoming a public health issue as birth rates continue in a sustained decline across Europe. Over the last 50 years, they have plummeted to reach an unprecedented low of 1.4 children per couple (Commission of the European Communities, 2009). In 2008, the European Parliament (2008) acknowledged for the first time that falling birth rates were a major cause of its population decline. Over mortality and migration, small family size is the major determinant of the future population number and composition in Europe (Maccheroni, 2007). Infertility affects one in six couples of childbearing age (Hull *et al.*, 1985), and male problems are responsible for 40% of these cases (Fleming *et al.*, 1995). One solution to the problem of reduced birth rates is to lessen the decline through the use of assisted reproduction technology (ART). Europe already performs ~60% of all ART treatments in the world (Nygren and Andersen, 2001) and in European countries between 1% and 6% (Andersen and Erb, 2006; RAND, 2006) of the births are currently aided by ART. Hence, ART has the potential to significantly influence adverse economic and demographic factors, and the European parliament has finally recognized that infertility treatment should be incorporated into the proposed population policy mix (European Parliament, 2008; Ziebe and Devroey, 2008). The European Parliament (resolution adopted by Parliament on 21 February 2008) calls on Member States to ensure the right of couples to universal access to infertility treatment. If implemented, this would be a major step forward since the majority of provision for infertility is currently in the private sector (except in Scandinavia and Belgium) with only those who can afford it having access to such services.

The next step forward is for clinicians to accept the need for, and scientists to work in partnership to devise, novel diagnostic and prognostic tests to improve the relatively modest ART success rates. Mean European 'take-home baby' rates still have room for improvement as they are 30.1% (Andersen *et al*., 2008) compared with 27.0% a decade ago (Land and Evers, 2003), although some countries are more successful than others (Van den Bergh *et al*., 2006). The UK national live birth rate for fresh cycles to women less than 35 years is 32.3% (Human Fertilization and Embryology Authority, 2003, 2007), although it was lower in 2000 (21.8%). If ART is to be included as a substantial part of the new population policy, there will need to be government-led and -funded demands for improvement of ART success rates. Male infertility has been long neglected and this is the area where most rapid progress could be made. However, this will force ART personnel to re-examine the assessment of male fertility potential and agree on improved prognostic sperm function tests with clinical relevance for each type of ART treatment.

Conventional semen analysis by light microscopic assessment of semen parameters (semen volume, sperm count, motility and morphology) is now recognized to be of limited value in the determination of the couples' fertility status (reviewed by Lewis, 2007). In contrast, sperm DNA testing has been increasingly recognized as a more promising test (Aitken and de Iuliis, 2007; Evenson *et al*., 2007; Zini *et al*., 2008). Measurement of sperm DNA damage is a useful biomarker for infertility with numerous studies showing its association with longer times to conceive compared with fertile couples (Spano *et al*., 2000), impaired embryo cleavage (Morris *et al*., 2002), higher miscarriage rates (Evenson *et al*., 1999) and also a significantly increased risk of pregnancy loss after IVF and ICSI (Zini *et al*., 2008). However, the implications of sperm DNA damage are even farther reaching. As sperm have few repair mechanisms (Jansen *et al*., 2001; Olsen *et al*., 2003; Aitken and Baker, 2006) and oocytes can only repair limited amount of damage (Ahmadi and Ng, 1999; Derijck *et al*., 2008), the damage to sperm DNA may affect the germ line for generations (Aitken and de Iuliis, 2007). Of even more concern than its ability to reduce fertility is the knowledge that sperm with oxidative DNA damage may still retain the potential to reach the oocyte, achieve fertilization and thereby contribute to mutations during embryonic development (Fraga *et al*., 1991) or even to loss of the fetus. If damaged sperm DNA is incorporated into the embryonic genome, it may lead to errors in DNA replication, transcription and translation during embryogenesis, contributing to a number of human diseases (Cooke *et al*., 2003) in not just one but subsequent generations (reviewed by Aitken *et al*., 2008). In particular, sperm DNA can impact on the short- and long-term health of children born by ART. Children conceived by ART, particularly ICSI, have a higher incidence of disease than those conceived spontaneously (Basatemur and Sutcliffe, 2008).Continuing into childhood, there is a strong association between poor sperm DNA integrity and diseases ranging from childhood cancers and leukaemias to autism (reviewed by Aitken and de Iuliis, 2007), especially aggravated by paternal smoking (Ji *et al*., 1997; Sorahan *et al*., 1997). A number of studies have shown major congenital malformations are present in 10% of ICSI children compared with 3% in spontaneously conceived counterparts (Lie *et al*., 2005; Sutcliffe and Ludwig, 2007; Katari *et al*., 2009; Williams and Sutcliffe, 2009; Woldringh *et al*., 2010), whereas other reviews suggest little difference in the health of the two groups (Ludwig *et al*., 2006). There is controversy surrounding the assessment and clinical value of DNA assessments; however, despite the current conflict in the literature (Barratt *et al*., 2010: Sakkas and Alvarez, 2010), studies are rapidly accumulating (reviewed by Aitken *et al*., 2008) to show that the link is through DNA damage to the father's sperm and that DNA damage is higher in ICSI patients (Bungum *et al*., 2007). Although there is much evidence associated with sperm DNA damage and poor ART outcomes, the tests have not been brought into clinical use.

Clinical thresholds to predict the chance of sperm populations achieving a clinical pregnancy (CP) have been established for the sperm chromatin structure assay (SCSA) (Bungum *et al*., 2007, reviewed by Evenson *et al*., 1999). A number of recent studies also show inverse relationships between fertility outcomes and DNA fragmentation (DF) using the terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labelling assay (TUNEL; Spano *et al*., 2000; Henkel *et al*., 2004; Tesarik *et al*., 2004). As yet, there are no clinical thresholds for the Comet assay (Lewis *et al*., 2004), although it is recognized to be more sensitive than other DNA damage tests (Leroy *et al*., 1996; Irvine *et al*., 2000) and is the only technique that allows the measurement of DNA damage in individual cells; particularly useful in a heterogeneous population such as sperm. The Comet assay measures both single- and double-strand DNA breaks using an alkaline pH method (Hughes *et al*., 1996; Donnelly *et al*., 2001). The Comet assay is highly reproducible (Hughes *et al*., 1997) and as it requires a much smaller number of cells (Hughes *et al*., 1996) for analysis than other tests, it is suitable for measures of testicular and oligozoospermic sperm samples where cells are scarce.

Oxidative stress (OS) has long been implicated as the major aetiological factor in sperm DNA damage. A low physiological level of reactive oxygen species (ROS) is accepted as necessary to maintain normal sperm function (Agarwal *et al*., 2003) but if ROS levels exceed physiological norms they lead to deteriorating function or reduced survival (Aitken and Baker, 2002). In contrast to somatic cells, sperm are very vulnerable to OS (Sies *et al*., 1992; Sies, 1993) owing to their unique membrane structures combined with limited antioxidants (Lewis *et al*., 1995) or protective enzymes. Not only does OS cause strand breaks but it also instigates deoxyribose damage, loss of bases or modifications to bases, such as 7,8-dihydro-8-oxo-2′-deoxoguanosine (8-OHdG), a modified base (MB) of the purine guanosine (Croteau and Bohr, 1997). Furthermore, such base modifications may also lead to discrete DNA strand breaks (Croteau and Bohr, 1997). Of the numerous oxidative MB (Croteau and Bohr, 1997), 8-OHdG is one of the most abundant and readily studied. Compared with other cell types, sperm exhibit much greater oxidative DNA damage as measured by 8-OHdG, $\sim 10^{-5}$ dG (Kodama *et al*., 1997), and higher levels of 8-OHdG have been observed in sperm from infertile compared with healthy subjects (Kodama *et al*., 1997; Shen *et al*., 1999) as well as an inverse correlation between sperm counts and 8-OHdG (Kodama *et al*., 1997; Ni *et al*., 1997; Shen *et al*., 1999; Xu *et al*., 2003). Therefore, the measurement of MB combined with DF assays gives an insight into potential, as well as existing, DF and may prove to enhance the prognostic usefulness of the current test.

In this study, we have used the alkaline Comet assay with and without the addition of formamidopyrimidine DNA glycosylase (FPG). This is a bifunctional DNA glycosylase recognizing oxidated purines, such as 8-OHdG, thereby converting MB into strand breaks which can be measured by the Comet assay (Collins, 2004). In order to determine both actual and potential DNA damage, we used Comet ± FPG and assessed its usefulness as a prognostic test.

Materials and Methods

Subjects

Men attending the Regional Fertility Centre, Royal Jubilee Maternity Service, Belfast, for infertility treatment between March 2008 and September 2009 were invited to participate in this study [$n = 230$ from IVF, mean (± SD) age 37.2 ± 0.3 years and $n = 130$ from ICSI, mean age 37.0 ± 0.5 years]. All subjects gave written informed consent for participation in this study, and the project was approved by the Office for Research Ethics Committees in Northern Ireland and the Royal Group Hospitals Trust Clinical Governance Committee. 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 (WHO, 1999). Sperm morphology was assessed according to WHO (1992) criteria. Semen analysis was performed within 1 h of ejaculation, following a period of incubation at 37°C to allow for liquefaction. After liquefaction, routine semen analyses were performed and subsequently semen was purified by density gradient centrifugation (DGC) using a two-step discontinuous Puresperm gradient (90–45%; Hunter Scientific Limited, UK). For each semen sample with a normozoospermic profile, the whole sample was layered on the top of 2 ml (90%) and 4 ml (45%) gradient and centrifuged at 250*g* for 20 min. For semen samples with less than normal WHO parameters, 1 ml of semen was layered on the top of 1 ml (90%) and 1 ml (45%) gradient and centrifuged at 100*g* for 20 min. The resulting sperm pellets were washed twice with Vitrolife G5 culture media (Vitrolife Inc., Goteborg, Sweden) and concentrated by centrifugation at 250*g* (normozoospermic) and 100*g* (subnormal) for 10 min and resuspended in fresh culture media (2 ml). Hence, two populations of sperm for each patient were used to measure DNA damage by the Comet assay that with the best fertilizing potential as used for their clinical treatments (DGC sperm) and the whole population (native semen).

ART procedures

All IVF cycles were performed according to the routine procedures (Donnelly *et al.*, 1998). Briefly, ovulation induction was achieved with recombinant FSH following a long protocol of pituitary desensitization with a GnRH analogue. HCG was administered when there were at least four follicles of diameter >17 mm, 36 h before oocyte retrieval. Mature, metaphase II oocytes obtained by vaginal ultrasound-guided aspiration were cultured in media [Vitrolife G5 sequential media series (Vitrolife Inc.)] at 37°C with 6% CO_2 in air. The ICSI procedure has been described in detail previously (Van Steirteghem *et al.*, 1993). In brief, a suspension of washed sperm was placed in polyvinylpyrrolidone (Vitrolife Inc.) and a free, motile sperm immobilized. The sperm was aspirated into the injection pipette tail-first and injected into an oocyte. Fertilization was recorded 12–16 h after injection. In each case, one or two embryos were transferred into the uterine cavity after an additional 24–48 h. Luteal phase support was provided by vaginally administered progesterone. An intrauterine pregnancy with fetal heart beat was confirmed by ultrasound 5 weeks after embryo transfer.

Single-cell gel electrophoresis (Comet) assay

Nuclear DF was assessed using an alkaline single-cell gel electrophoresis (Comet) assay as modified previously by our group (Hughes *et al.*, 1997; Donnelly *et al.*, 1999). Our previous study has reported an intra-assay coefficient variation of 6% for this assay (Hughes *et al.*, 1997).

FPG treatment

Of the MB, 8-OHdG is the most commonly studied biomarker and is often selected as being representative of oxidative DNA damage owing to its high specificity, potent mutagenicity and relative abundance in DNA (Floyd, 1990). We used the protein FPG, a bacterial repair enzyme isolated from *Escherichia coli*, which recognizes and excises 8-OHdG generated by ROS. The FPG enzyme extract was purified from *E. coli* ER 2566 strain harbouring the pFPG230 plasmid, as described previously (Boiteux *et al.*, 1990; Olsen *et al.*, 2003). The extract has been shown to possess affinities towards the various DNA base modifications known to be recognized by pure FPG (Dr S. Sauvaigo, personal communication).

The catalytic activity of FPG involves a three-step process: (i) hydrolysis of the glycosidic bond between the damaged base and the deoxyribose, (ii) incision of DNA at basic sites leaving a gap at the 3′- and 5′-ends by phosphoryl groups and (iii) removal of terminal deoxyribose 5′-phosphate from 5′-terminal site to excise the damaged base, as shown by Kuznetsov *et al.* (1998). To analyse MB, FPG (stock concentration of 19.14 mg/ml, diluted to a final concentration of 1 μg/ml) was added to sperm to introduce breaks at sites of MB during decondensation by lithium 3,5-diiodosalicylate and incubated at 37.0°C for 90 min. Our previous study (Hughes *et al.*, 1997) showed an intra-assay coefficient variation of 6% for the Comet assay. The overall SEM for all IVF/ICSI samples in this study without FPG is low (~4%) and is not increased by FPG, suggesting that variation linked to the addition of FPG is of minor importance.

Data and statistical analysis

Data were analysed using the Statistical Package for the Social Sciences (SPSS 15) for Windows (SPSS Inc., Chicago, IL, USA). Demographic details of couples are given in Table I according to the treatment (IVF or ICSI) and outcome. Our primary outcome for each treatment was the effect of DNA damage (analysed by Comet ± FPG) on CP, evaluated in both native semen and DCG sperm by logistic regression. The key outcome from the model derived above is individual posterior probabilities of a positive CP. We tested the performance of our prognostic model by calculating the *c*-statistic, which is identical to the area under the receiver operating characteristic (ROC) curve. Essentially, all possible pairs of individuals where one is pregnant and one is not pregnant were considered. Then, the number of such pairs where the posterior probability for the pregnant couple is higher than the posterior probability for the non-pregnant couple was counted: this was defined as the *c*-statistic. A null performance of the model would result in a *c*-statistic of 0.5.

Secondary outcomes were fertilization rate (FR) and embryo cumulative score (ECS). The FR was calculated as the percentage of all fertilized oocytes for IVF, and the percentage of metaphase II oocytes with two pronuclei for ICSI. The ECS was calculated for 153 couples who had embryo transfers on Day 3, by multiplying embryo grade (A = 4, B = 3, C = 2 and D = 1) by the number of blastomeres for each embryo and where a patient had more than one embryo, a mean across embryos was calculated to obtain the total quality of all embryos generated (ECS_1) or embryos transferred (ECS_2). Use of ECS, as opposed to number of high-quality embryos, allows for quantification of the number and quality of blastomeres, making associations more precise. Relationships between sperm DF and the FR and ECS were compared using the Spearman rank correlation test. Associations between conventional semen parameters and DF and MB were also assessed using the Spearman rank correlation test. To determine the extent of damage contributed by MB, we compared existing DNA strand breaks with total strand breaks

Table I Demographic data for couples undergoing ARTs.

	IVF ($n = 230$)				ICSI ($n = 130$)			
	Pregnant	**Non-pregnant**	**CI**	***P*-value**	**Pregnant**	**Non-pregnant**	**CI**	***P*-value**
Cycles included (*n*)	39	180	—	—	34	82	—	—
Female age (years)	34.4 ± 4.3	37.5 ± 5.7	−11.3 to 5.1	NS	34.9 ± 3.7	34.9 ± 5.2	−1.9 to 1.9	NS
Number of previous treatments	1.5 ± 0.9	1.4 ± 1.2	−0.4 to 0.5	NS	1.8 ± 0.9	1.7 ± 1.0	−0.4 to 0.5	NS
Oocytes retrieved	10.5 ± 6.4	8.2 ± 5.2	0.4–4.2	0.021	10.2 ± 5.0	8.1 ± 4.9	0.3–4.0	NS
Oocytes fertilized (2 pronuclei)	6.9 ± 3.9	4.9 ± 4.0	0.7–3.5	0.004	6.7 ± 3.8	8.5 ± 3.2	−0.1 to 2.5	NS
Fertilization rate (%)	72.6 ± 20.6	62.5 ± 31.2	−0.5 to 20.7	0.062	77.6 ± 20.5	79.7 ± 18.4	−10.1 to 5.8	NS
Embryos transferred	1.9 ± 0.2	1.6 ± 0.7	0.1–0.6	0.013	1.9 ± 0.4	1.8 ± 0.4	−0.1 to 0.2	NS
Total embryo cumulative score (ECS_1)	18.1 ± 10.9	13.3 ± 9.3	0.9–7.1	0.012	12.0 ± 5.5	11.4 ± 4.9	−1.6 to 3.0	NS
Transferred embryo cumulative score (ECS_2)	43.8 ± 17.8	31.0 ± 12.5	4.5–19.9	0.002	51.1 ± 2.5	42.2 ± 2.3	0.3–13.7	0.049
Male age (years)	36.1 ± 4.9	37.4 ± 5.0	−3.1 to 0.4	NS	38.3 ± 4.6	36.4 ± 4.9	0.01–3.8	NS
Semen volume (ml)	3.1 ± 1.4	3.5 ± 2.4	−1.2 to 0.4	NS	3.4 ± 2.2	3.6 ± 2.8	−1.3 to 0.9	NS
Sperm concentration (10^6 ml^{-1})	63.7 ± 36.0	67.4 ± 40.7	−17.9 to 10.5	NS	42.3 ± 49.1	30.6 ± 34.0	−5.8 to 29.2	NS
Total sperm output (10^6)	199.3 ± 143.2	233.6 ± 392.5	−183.6 to 75.1	NS	157.5 ± 220.8	115.5 ± 148.4	−37.4 to 121.4	NS
Motility (%)	56.1 ± 20.9	54.8 ± 17.1	−5.1 to 7.7	NS	45.9 ± 21.0	43.8 ± 22.3	−7.5 to 11.7	NS
Normal morphology (%)	28.5 ± 15.2	24.7 ± 9.4	−0.01 to 7.6	0.05	20.4 ± 9.9	18.1 ± 11.2	−2.4 to 6.9	NS

Values are the mean ± SD. NS, non-significant; CI, 95% confidence interval.

(i.e. including converted MB) using a paired sample *t*-test. A *P*-value <0.05 was considered statistically significant.

To compare the prognostic ability of the different sperm DNA damage variables, we ran logistic regression models with pregnancy (yes/no) as outcome and with each of four DNA damage markers individually as explanatory variables: the markers were DF measured by the alkaline Comet assay in native semen and in populations of sperm following DGC, with and without the addition of MB. We used the 230 IVF cycles to determine thresholds of DF (not including MB) in native and DCG sperm. Predicted probabilities of a pregnancy were obtained to achieve CP with 80% power. From these, we estimated thresholds where the predicted probability of a positive pregnancy was equal to 0.1 (ED10). Odds ratios (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. Separate models were constructed for IVF and ICSI treatment groups. CIs for ROC, which include the value 0.5, are statistically indistinguishable.

Results

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

Semen samples from couples who achieved a CP were compared with those who were unsuccessful (Table I). Abstinence times did not differ between the groups. No significant differences were observed in semen volume, sperm concentration, total sperm output and percentage motility.

Correlations between DF, FR and embryo quality assessed by the alkaline Comet assay following IVF treatment

There was no decrease in FR as DNA damage of native semen increased (data not shown, $P > 0.05$). In contrast, there was a decrease in FR as sperm DF increased in DGC sperm: 0–20% and 21–40% DF were associated with higher FR (69.9 ± 3.7% and 66.4 ± 4.2%, respectively) compared with an FR of 54.4 ± 6.0% when DNA damage was 61–100% in DGC sperm ($P < 0.05$, Fig. 1). There was also a decrease in ECS as DF increased, both in native semen and in DGC sperm. The cumulative embryo score (ECS_1) for all the embryos generated showed a significant decrease, when DNA damage was greater than 60% in the native semen. The ESC_1 was 15.5 ± 2.6% in the group where sperm DF was 0–20% reducing to 10.7 ± 1.5% in the group where DF was 61–100% ($P = 0.020$) in the native semen. The ECS_1 was only 7.3 ± 2.5% where sperm DF was 61–100% in DGC sperm ($P = 0.032$; Fig. 2). Similarly, the ECS_2 showed a decrease when DF was greater than 60% compared with that below 20% DNA damage (38.1 ± 6.6 and 26.7 ± 3.8, respectively, $P = 0.007$) in the native semen and (18.7 ± 5.9 and 34.1 ± 3.60, respectively, $P = 0.034$; Fig. 3) in the DGC sperm. Pregnant couples had higher mean ECS_1 ($P = 0.012$) and ECS_2 ($P = 0.002$) than non-pregnant couples (Table I). There was a correlation between FR and sperm motility ($P = 0.014$) but no significant correlations were seen between any other semen parameter and FR, ECS_1, ECS_2 or CP.

Sperm DF of pregnant and non-pregnant couples following IVF

Using the Comet assay, the mean percentage of sperm DF was significantly higher in sperm from non-pregnant couples ($n = 180$) compared with that from pregnant couples ($n = 39$) undergoing IVF in both the native semen (51.7 ± 23.6 versus 39.5 ± 17.9; $P = 0.004$) and the DGC sperm for clinical use (36.8 ± 21.6 versus 26.9 ± 14.6; $P = 0.01$) (Table II). Using the threshold values of 56% for the native semen and 44% for the DGC sperm, we calculated OR and CI of 4.52 (1.79–11.92) and 6.20 (1.74–26.30), respectively, for CP (Fig. 4; Tables III and IV).

Sperm DF, FR, embryo quality and pregnancies following ICSI treatment

The ECS_2 had a significantly higher score for pregnant couples (51.1 ± 2.5) than non-pregnant couples (42.2 ± 2.3; $P = 0.049$). Sperm

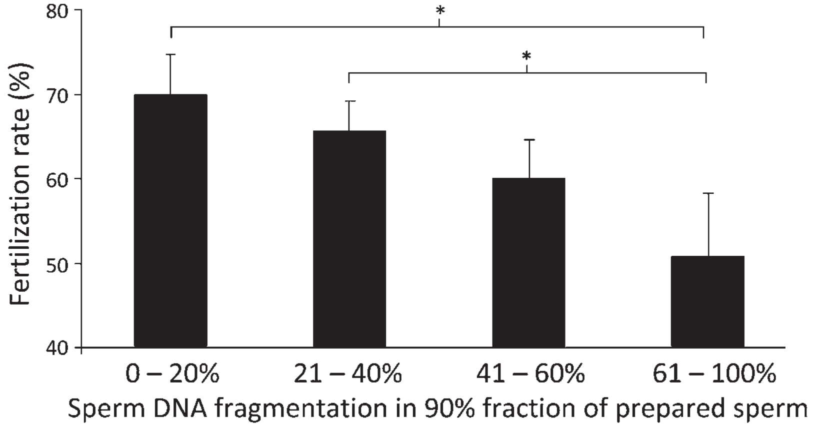

Figure 1 Bar chart showing decrease in fertilization rate (FR) with increase in DNA damage in the sperm prepared using DGC (sperm), for patients undergoing IVF. Values are mean ± SEM, *$P < 0.05$, $n = 222$.

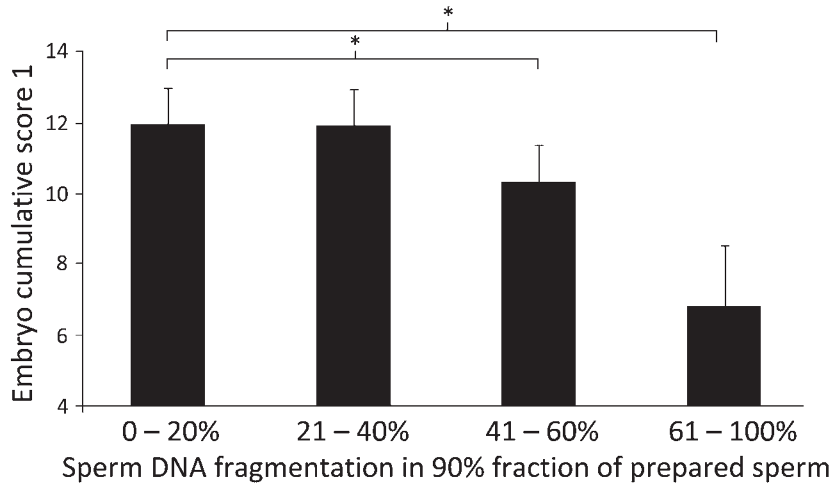

Figure 2 Bar chart showing decrease in cumulative embryo score of total embryos generated with increase in DNA damage in the DGC sperm, for patients undergoing IVF. Values are mean ± SEM, $*P < 0.05$, $n = 153$.

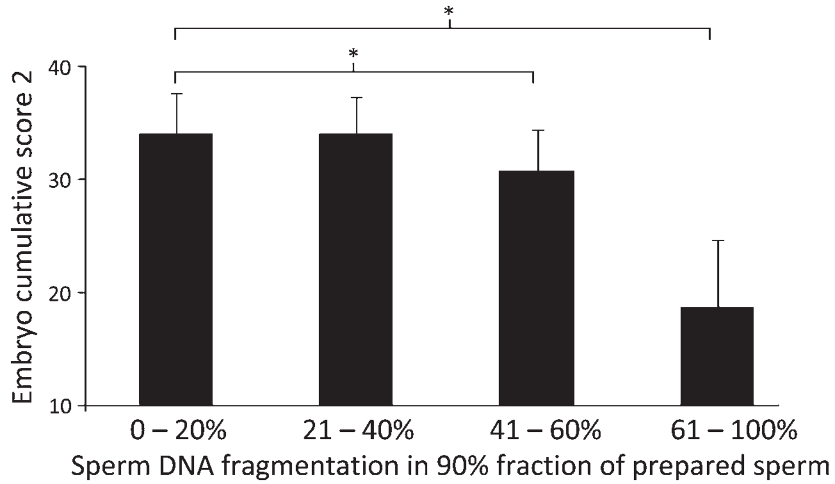

Figure 3 Bar chart showing decrease in cumulative embryo score of transferred embryos with increase in DNA damage in the DGC sperm, for patients undergoing IVF. Values are mean ± SEM, $*P < 0.05$, $n = 153$.

from partners of couples undergoing ICSI who failed to achieve a CP tended to have more DF than sperm from pregnant couples (net increase of +8.3% native semen, $P = 0.109$ and +6.2% DGC sperm, $P = 0.243$) (Table II). There was no correlation between sperm DNA damage and FR, or ECS_1 or ECS_2.

The relationship between total DNA damage and IVF and ICSI outcomes after conversion of MB to DNA strand breaks by FPG

A significant increase in DNA damage was detected after treatment with the DNA glycosylase FPG in both native and DGC samples ($P < 0.0001$; Table V). The variation in damage (with FPG) ranged from 0% to 47% in the native and 0% to 45% in the DGC sperm. In the IVF patients, addition of the FPG enzyme showed a significant increase in DF in sperm from non-pregnant ($n = 63$) compared with that from pregnant couples ($n = 10$) in the native semen (with a net increase of +17.1%; $P = 0.009$) and in the DGC sperm (a net increase of +13.8%; $P = 0.045$) (Table II). Similarly, in ICSI couples, when MB were included, the DNA damage between pregnant and non-pregnant couples was markedly different (with a net increase of +16.8% native semen, $P = 0.008$ and +15.5% DGC sperm, $P = 0.024$) in contrast to Comet without FPG, where there was no significance (Table II).

The prognostic value of DNA damage (strand breaks plus adducts) testing

We tested the performance of our prognostic model by calculating the area under the ROC curve (Table II). Essentially, all possible pairs of individuals where one is pregnant and one is not pregnant

Table II Comparison of DF between pregnant and non-pregnant couples after IVF and ICSI treatments.

	IVF					ICSI				
	Pregnant couples	**Non-pregnant couples**	**Difference (95% CI)**	***P*-value**	**ROC (95% CI)**	**Pregnant couples**	**Non-pregnant couples**	**Difference (95% CI)**	***P*-value**	**ROC (95% CI)**
n	39	180	—	—	—	34	82	—	—	—
DF in native semen (%)	39.5 ± 17.9	51.7 ± 23.6	12.2 (−15.9, −4.0)	0.004	0.648 (0.561, 0.735)	58.9 ± 25.7	67.2 ± 25.6	8.3 (−18.5, 1.9)	0.109	0.601 (0.488, 0.713)
DF in DGC sperm (%)	26.9 ± 14.6	36.8 ± 21.6	9.9 (−17.5, −2.4)	0.010	0.629 (0.542, 0.717)	45.5 ± 24.5	51.7 ± 27.0	6.2 (−16.7, 4.2)	0.243	0.572 (0.461, 0.683)
n	10	63	—	—	—	15	38	—	—	—
DF in native semen after FPG treatment (%)	54.7 ± 4.9	71.8 ± 19.1	17.1 (−29.7, −4.4)	0.009	0.776 (0.643, 0.910)	63.1 ± 23.6	79.9 ± 18.7	16.8 (−29.2, −4.6)	0.008	0.704 (0.537, 0.872)
DF in DGC sperm after FPG treatment (%)	42.2 ± 6.5	56.0 ± 19.9	13.8 (−27.4, −0.3)	0.045	0.693 (0.524, 0.862)	50.0 ± 22.2	65.5 ± 21.7	15.5 (−28.9, −2.1)	0.024	0.717 (0.555, 0.878)

Values are expressed as the mean ± SD. ROC, receiver operating characteristic (area under, cm^2); DGC, density gradient centrifugation; FPG, formamidopyrimidine DNA glycosylase enzyme.

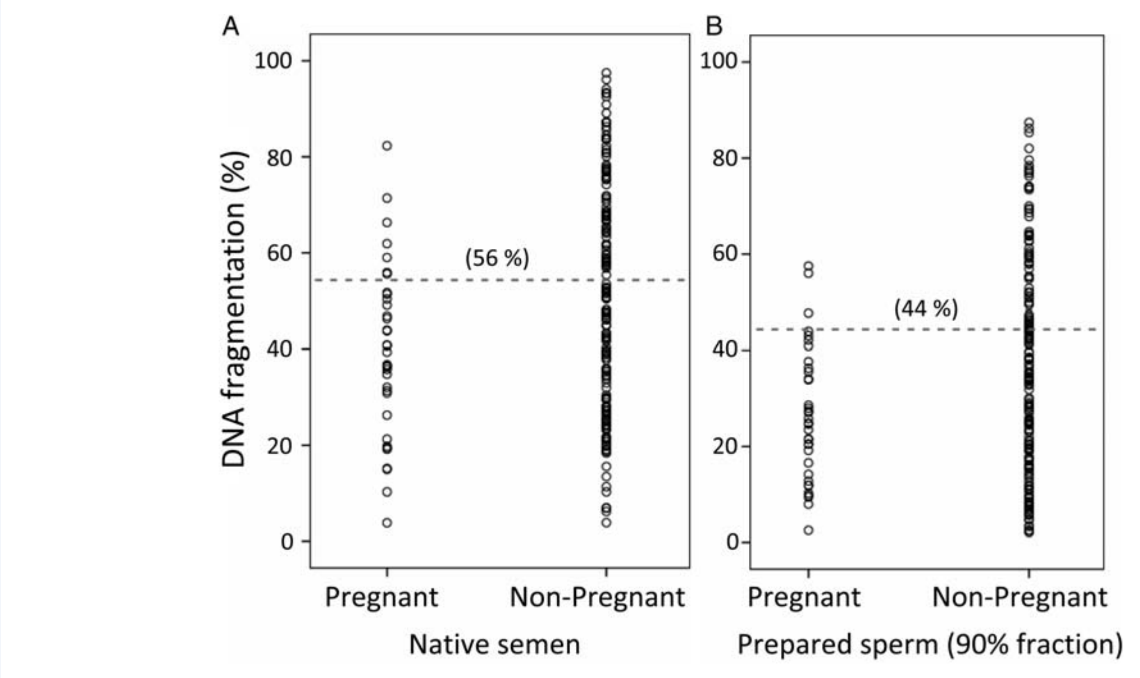

Figure 4 Scatter plot showing DNA fragmentation (DF) measured by Comet in the native and DGC sperm according to their pregnancy outcome in IVF. $n = 219$.

were considered. The analysis for IVF CP showed the area under the curve is 0.648 cm^2 ($P = 0.006$) for the native semen and 0.629 cm^2 ($P = 0.016$) for the DGC sperm from pregnant compared with non-pregnant couples, respectively. When MB were included the area under the ROC curve increased to 0.776 cm^2 ($P = 0.005$) for the native semen and 0.693 cm^2 ($P = 0.05$) for the DGC sperm. In the ICSI group, for total DNA damage (including MB), a significant difference was observed between pregnant and non-pregnant couples. The ROC analysis for ICSI when MB were included also increased the area under the ROC curve in both the native semen and the DGC sample

Table III OR on ART outcome in IVF and ICSI cycles using a cut-off value of 56% in the native semen.

	IVF			ICSI		
	<56%	>56%	OR (95% CI)	<56%	>56%	OR (95% CI)
Cycles started	127	97	—	47	80	—
Biochemical pregnancies (*n*, % per cycle)	44 (34.6)	18 (18.6)	2.58 (1.31–5.12)	23 (48.9)	31 (38.8)	1.18 (0.52–2.68)
Clinical pregnancies (*n*, % per cycle)	32 (25.2)	7 (7.2)	4.52 (1.79–11.92)	20 (42.6)	24 (30.0)	1.97 (0.81–4.77)
Deliveries to date (*n*, % per cycle)	27 (21.3)	2 (2.1)	10.13 (0.74–294.05)	17 (36.2)	15 (18.8)	5.25 (1.15–25.7)
Early pregnancy loss (*n*, % per cycle)	2 (1.6)	2 (2.1)	16.5 (0.95–424.73)	3 (6.4)	5 (6.3)	2.95 (0.49–19.16)

Table IV OR on ART outcome in IVF and ICSI cycles using a cut-off value of 44% in the DGC sperm.

	IVF			ICSI		
	<44%	>44%	OR (95% CI)	<44%	>44%	OR (95% CI)
Cycles started	158	66	—	54	72	—
Biochemical pregnancies (*n*, % per cycle)	51 (32.3)	11 (16.6)	2.32 (1.07–5.14)	25 (46.3)	26 (36.1)	1.53 (0.70–3.34)
Clinical pregnancies (*n*, % per cycle)	36 (22.8)	3 (4.5)	6.20 (1.74–26.30)	24 (44.4)	20 (27.8)	2.08 (0.93–4.68)
Deliveries to date (*n*, % per cycle)	28 (17.7)	1 (1.5)	57.00 (3.47–1794.29)	19 (35.2)	9 (12.5)	0.90 (0.22–3.61)
Early pregnancy loss (*n*, % per cycle)	2 (1.3)	2 (3.0)	84.00 (2.89–14 248.30)	4 (7.4)	4 (5.6)	2.38 (0.37–15.89)

Table V Comparison of DF in the native semen and the DGC sample according to the treatments.

ART treatment	*n*	Test	Native semen	DGC sperm	*P*-value
IVF	230	Comet	49.5 ± 1.6	35.2 ± 1.4	0.0001[a]
	73	Comet + FPG	74.6 ± 2.9	59.8 ± 3.1	0.0001[a]
P-value			<0.0001[b]	<0.0001[b]	
ICSI	130	Comet	64.0 ± 2.3	49.1 ± 2.3	0.0001[a]
	53	Comet + FPG	79.4 ± 2.3	54.1 ± 2.4	0.0001[a]
P-value			<0.0001[b]	<0.0001[b]	

Values are the mean ± SEM.
[a] *P*-value of comparison between native semen and DGC sperm.
[b] *P*-value of comparison between Comet and Comet + FPG.

Table VI Comparison of cut-off values predicting CP in the native semen (56%) and the DGC sample (44%).

	IVF		ICSI	
	Native (%)	DGC (%)	Native (%)	DGC (%)
Sensitivity	82.1	92.3	47.2	54.6
Specificity	49.7	34.6	68.8	63.4
PPV	26.7	22.8	40.5	44.4
NPV	92.6	95.5	74.3	72.2
RR	3.6	5.0	1.6	1.6

PPV, positive predictive value; NPV, negative predictive value; RR, relative risk.

compared with Comet alone, 0.704 cm^2 compared with 0.601 cm^2 ($P = 0.015$) and 0.717 cm^2 compared with 0.572 cm^2 ($P = 0.005$), respectively, again indicating the improved prognostic ability with MB. Measurement of DF in native semen and DGC sperm had a higher sensitivity but lower specificity in IVF than ICSI treatment (Table VI). The threshold value showed a high negative predictive value (93% and 95%) for IVF CP using both native and DGC sperm, respectively. The positive predictive value for IVF and ICSI success with native sperm was less robust, being 27% and 40%, respectively.

Discussion

In this study, the predictive value of sperm DF in native and DCG sperm on IVF and ICSI outcomes was assessed in a cohort of 360 ART patients using the alkaline Comet assay. The predictive power was significantly increased by using a modified Comet assay allowing oxidated purines to be measured, by converting such base modifications into strand breaks by means of the DNA glycosylase, FPG. We noted a relationship between existing DF and ART outcomes (FR, ECS_1, ECS_2 and CP) in IVF cycles but not in ICSI cycles. However, when the MB were converted into strand breaks, an inverse relationship was also observed in ICSI cycles. In the IVF group, using a threshold value of 56% and 44% DF (for native semen and DGC sperm, respectively, without FPG treatment), there was a significant decrease in pregnancy rates in the high DF group.

Male infertility diagnosis is still based on the conventional semen analysis, despite its prognostic and diagnostic limitations for the infertile couple (reviewed by Tomlinson *et al.*, 1999; Lewis, 2007). Some studies have shown relationships between DF and sperm concentration (Tomlinson *et al.*, 2001), normal morphology and progressive motility (Larson-Cook *et al.*, 2003) or the absence of immature sperm (Virro *et al.*, 2004) in native semen. Our data support those of Frydman *et al.* (2008) and Greco *et al.* (2005) in showing few correlations between conventional semen parameters and DF and is in conflict with the study reported by Irvine *et al.* (2000) where sperm DNA damage assessed by the Comet assay was closely associated with semen quality; in particular with sperm concentration. Here, in 68% of IVF patients, semen profiles were normozoospermic according to the WHO criteria, yet almost half of those men had DF above our threshold value of 48%. In the ICSI group, 34% had normal semen parameters (these couples had previously had IVF treatment: 47% had failed fertilization and 53% had not achieved a pregnancy), although 54% had DF above 50%. Thus, no strong relationships were found between semen analysis parameters and DF. Although conventional parameters have been shown to have no correlation with ICSI outcome (Nagy *et al.*, 1995, 1998), these are still the characteristics by which sperm are chosen clinically, yet in this study we have again shown that these are not necessarily the sperm with the best DNA. Since sperm DNA tests show more promise, it is urgent to refine these tests until they are sufficiently robust for routine clinical use.

Sperm DNA damage has been closely associated with numerous indicators of reproductive health, including FR, ECS, implantation and spontaneous miscarriage (Lewis and Aitken, 2005; Frydman *et al.*, 2008) using several techniques to assess sperm DNA damage. Of these, the Comet assay under alkaline and neutral conditions, TUNEL assay and SCSA (reviewed by Evenson *et al.*, 2002; Agarwal and Said, 2003) have been shown to be most robust. Each of these tests assesses different aspects of DNA damage. The SCSA is based on partial acid-induced denaturation and staining with acridine orange, and analysis of the staining pattern of each cell using flow cytometry. On the other hand, TUNEL assay is a direct method for the assessment of DF, by quantifying the incorporated dUTP at double-strand DNA breaks catalysed by terminal deoxynucleotidyl transferase (Martins *et al.*, 2007). The alkaline Comet assay assesses double- and single-strand DNA breaks and alkali labile sites. It has been used *in vitro* and *in vivo* in a wide variety of mammalian cells (Singh *et al.*, 1988; Tice *et al.*, 1990; Olive *et al.*, 1998) employing a number of different genotoxic stimuli including UV radiation, carcinogens, radiotherapy and chemotherapy (Fairbairn *et al*., 1995). The alkaline Comet assay is highly reproducible (Hughes *et al*., 1997) with greater sensitivity than alkaline elution or nick translation assays even without prior chromatin decondensation (Leroy *et al*., 1996; Irvine *et al*., 2000). The Comet assay can detect damage equivalent to as few as 50 single-strand breaks per cell: another of its unique and powerful features is the ability to characterize the responses of a heterogeneous population of cells by measuring DNA damage within individual cells as opposed to just one overall measure of damaged cells versus undamaged cells, as in the TUNEL. A further advantage is that, unlike the TUNEL and SCSA which detect primarily breaks in histone-associated chromatin, the Comet assay has a broader use in detecting breaks in both protamine and histone-bound chromatin equally. One drawback of the Comet is that it requires trained researchers to perform it optimally and results can vary from lab to lab. Like the SCSA, the Comet assay would benefit from the standardized protocols and instruction from researchers who have used it extensively (e.g. those in the Robaire or Lewis laboratories).

The influence of DF on FR in assisted reproduction is still controversial since paternal DNA is not believed to influence this early fertility checkpoint. In this study, a negative correlation was observed between sperm DF and FR in both native and DGC sperm. We also observed a significant decrease in FR above 60% DF in native semen. Similarly, DGC sperm showed a significant decrease in FR with increase in DF (Fig. 1). This is in agreement with a number of studies that show a marked negative correlation between DF and FR in IVF using the TUNEL assay (Sun *et al*., 1997; Host *et al*., 2000; Benchaib *et al*., 2003; Huang *et al*., 2005; Payne *et al*., 2005; Borini *et al*., 2006; Bakos *et al*., 2008). Our data do not confirm those of studies using the SCSA that show no significant association of DF with FR (Larson *et al*., 2000; Larson-Cook *et al*., 2003). The adverse effects of DF seen here may be expected since abnormal chromatin packing in sperm is associated with high DNA damage (Simon, Lewis and Oliva, unpublished results) 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 negative correlation between DF and the cumulative embryo score of both ECS_1 and ECS_2, and this relationship is true for both native and DGC sperm. A similar association between sperm DF and poor ECS after IVF was reported by many groups (Host *et al*., 2000; Tomsu *et al*., 2002; Seli *et al*., 2004; Tesarik *et al*., 2004; Virro *et al*., 2004; Muriel *et al*., 2006). Our results contrast with those of studies showing no significant association of ECS and DF (Larson *et al*., 2000; Larson-Cook *et al*., 2003; Payne *et al*., 2005; Benchaib *et al*., 2007; Bungum *et al*., 2007; Bakos *et al*., 2008; Frydman *et al*., 2008). Van Royen *et al*. (2003) concluded that poor quality blastomeres can lead to cleavage stage arrest *in vitro* and are associated with a lower implantation rate. The impact of fragmented paternal DNA became more obvious when the embryonic genome was activated (Braude *et al*., 1988) giving the so-called 'late paternal effect' (Tesarik *et al*., 2004). Again this shows promise as a useful clinical biomarker since as sperm DNA damage increases, ECS_2 decreases and this is followed by a reduced likelihood of a successful CP ensuing (Sun *et al*., 1997; Morris *et al*., 2002; Tesarik *et al*., 2004). In contrast, in the ICSI group, there was no correlation between DF and FR. This is not surprising because the ICSI technique bypasses the requirement for sperm to penetrate the oocyte naturally. These data support studies where FR in ICSI was not influenced by sperm DF (Borini *et al*., 2006; Bungum *et al*., 2007; Bakos *et al*., 2008). However, again the literature is in conflict with numerous other studies reporting an inverse relationship between FR and DF (Lopes *et al*., 1998; Henkel *et al*., 2003; Huang *et al*., 2005; Payne *et al*., 2005; Muriel *et al*., 2006; Benchaib *et al*., 2007). Further, we did not observe a correlation between DF measured by the Comet assay and ECS_1 in ICSI cycles. Our results support the belief that DNA damage in the sperm is not important at this early stage, since 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 (Braude *et al*., 1988).

The transfer of good-quality embryos is a major determinant of CP rates with IVF and ICSI (Scott, 2003; Terriou *et al*., 2007). In our study, sperm DF above 60% was associated with poorer $ECS_{2,}$ and decreasing CP in IVF but not in ICSI treatments. In IVF, successful couples had a significantly higher quality of transferred embryo (ECS_2) than unsuccessful couples. However, in ICSI, the ECS_2 did not differ following insemination by sperm with high or low DF. The impact of sperm DF depends on the extent of damage in the sperm and ability of the egg to repair that damage (Gandini *et al*., 2004). It may be that since the primary reason for these couples' infertility is defined sperm problems, their oocytes are normal and are capable of repairing sperm DNA damage (Bungum *et al*., 2007; Ozmen *et al*., 2007). The age of the female partner has long been recognized as a significant factor in a couples' fertility. It influences pregnancy rates after vasectomy reversal (Gerrard *et al*., 2007), treatment of male infertility by ICSI and even the treatment of azoospermia by ICSI with surgically retrieved sperm (Silber *et al*., 1997). However, the data here are not an age-related phenomenon, as the ages of successful and unsuccessful women in both IVF and ICSI were similar (Table I). Another possibility is that laboratory conditions for sperm during ICSI are less deleterious than those for IVF. During ICSI, the sperm spend less time in culture media (Bungum *et al*., 2007) before injection into the protected environment of the oocyte and may therefore have less exposure to further oxidative damage that can occur *in vitro* (Dalzell *et al*., 2003; Agarwal *et al*., 2006). This possibility is supported by our data, where the addition of oxidative DNA damage, as indicated by converted MB, significantly enhances the value of the test for determining pregnancy end-points. Another option is that long periods in culture media (as more often occur in IVF) may lead to imprinting defects (Gosden *et al*., 2003) which could in turn impact adversely on CP rates. Since our knowledge is currently so limited as to which types of sperm DNA damage are irreparable and deleterious to reproduction, we can only speculate on this issue.

In our study, couples undergoing IVF had lower DF than those couples undergoing ICSI (26.9 ± 14.6% versus 45.5 ± 24.5%, respectively) and attained successful CP (Table II). In IVF, DF had a significant deleterious impact on CP (Larson *et al*., 2000; Tomlinson *et al*., 2001; Duran *et al*., 2002; Larson-Cook *et al*., 2003; Saleh *et al*., 2003; Virro *et al*., 2004; Frydman *et al*., 2008). In contrast to our study, others have found no correlation between DF and CP (Host *et al*., 2000; Morris *et al*., 2002; Tomsu *et al*., 2002; Benchaib *et al*., 2003, 2007; Gandini *et al*., 2004; Huang *et al*., 2005; Payne *et al*., 2005; Boe-Hansen *et al*., 2006; Borini *et al*., 2006; Muriel *et al*., 2006., Bakos et al., 2008; Lin et al., 2008). These differences in results may be arise from variations in the assay conditions and author imposed threshold values. The threshold level for TUNEL assay varies between 4% (Host et al., 2000; Huang et al., 2005), 10% (Borini et al., 2006), 15% (Benchaib et al., 2007), 20% (Benchaib et al., 2003; Seli et al., 2004) and 35% (Frydman et al., 2008) and for SCSA, 20% (Boe-Hansen et al., 2006), 27% (Larson et al., 2000; Larson-Cook et al., 2003) and 30% (Evenson et al., 1999; Virro et al., 2004; Payne et al., 2005; Zini et al., 2005), which illustrates that there are no standardized laboratory protocols for TUNEL assay. The clinical usefulness of a test is usually based on OR which in turn are based on threshold values which vary enormously (Collins et al., 2008) depending on the assay, preparation and scientific choice. The threshold value most commonly used is that drawn from a study by Evenson et al. (1999) where 165 presumed fertile couples, none with >30% DNA damage, achieved a CP and the conclusion was therefore that >30% DF was a threshold not considered compatible with fertility. However, this threshold may or may not be appropriate for couples undergoing IVF or ICSI and may differ for sperm from native and DGC populations. In this study, two threshold values have been used to calculate ORs, 56% for native sperm and 44% for the DGC sperm. These high values (relative to the SCSA and TUNEL) are related to the sensitivity of the Comet assay in that following lysis and decondensation, all double- and single-strand breaks and alkali labile sites are revealed in contrast to other assays where perhaps only peripheral DNA damage is determined. As viability testing is not included in our standard semen analysis, another reason for the high threshold for native sperm may be the inclusion of some non-viable cells.

In ICSI cycles, in contrast to IVF, we did not obtain a significant association between DF of native or DGC sperm and CP using the Comet assay without FPG (Table II), which is in agreement with many studies (Host et al., 2000; Morris et al., 2002; Benchaib et al., 2003, 2007; Bungum et al., 2004, 2007; Gandini et al., 2004; Greco et al., 2005; Huang et al., 2005; Payne et al., 2005; Zini et al., 2005; Boe-Hansen et al., 2006; Muriel et al., 2006; Lin et al., 2008). As before, the literature is divided and, in contrast to our results, there are also studies showing a significant decrease in CP with increase in DF (Larson et al., 2000; Larson-Cook et al., 2003; Saleh et al., 2003; Virro et al., 2004; Borini et al., 2006; Bakos et al., 2008; Bungum et al., 2008). However, our data support the hypothesis that ICSI is able to compensate for existing DNA strand breaks as well as inadequate conventional sperm parameters (Ozmen et al., 2007; Bungum et al., 2008).

A major cause of sperm DNA damage is OS, caused by the generation of the ROS from contaminating leucocytes, defective sperm and antioxidant depletion (Lewis et al., 1995; Garrido et al., 2004). In addition to damage caused by creating strand breaks, we measured, for the first time, additional oxidative damage by excising MB to make them measurable by the Comet assay. When we converted oxidized purines into strand breaks in both IVF and ICSI couples ($n = 126$), an increase in damage of 15.9 ± 1.3% was observed in native semen and 16.7 ± 1.4% in DGC sperm. By including MB, a strong association emerged between DF and CP rates in ICSI as well as increasing the sensitivity of detection in IVF. This shows the importance of including MB in potential prognostic tests for male infertility rather than focusing on existing strand breaks alone. Earlier studies had reported that in the measurement of MB (Horak et al., 2003a), 8-OHdG (Ni et al., 1997) is an important biomarker to investigate DNA damage and human infertility. MB are also known to increase in embryos of smoking couples (Zenzes et al., 1999). Recently, Horak et al. (2007) reported that sperm MB impairs FR during ICSI. Horak et al. (2003a) showed fertile individuals and patients with male-factor infertility differed significantly with respect to the level of bulky MB. A significant negative correlation is obtained between MB (Horak et al., 2003b), 8-OHdG (Ni et al., 1997) and semen quality in patients with an impaired fertility. Horak et al. (2003b) showed the level of bulky MB in sperm is positively associated with amounts of leucocytes in semen and also higher in semen of infertile subjects. By measuring both the DNA strand breaks and the FPG sensitive sites in human sperm, we increased the prognostic value of the Comet test. Since this study shows that a significant proportion of DNA damage is specifically a result of OS, it highlights the possibility of antioxidant therapy to protect sperm DNA prior to ART treatment.

Given the rigorous sperm selection that occurs naturally and repeatedly prior to fertilization (reviewed by Oehninger, 2000), and learning from the elegant studies of Harrison (1998) that a small minority of the unselected sperm population in semen may be normal by each assessment, it is important to examine subpopulations as well as the whole sperm population of the ejaculate. There is debate as to whether DGC isolates a subpopulation of sperm with less DF: this study supports previous work by our group (Donnelly et al., 2000) and also a report from Morrell et al. (2004) that this is indeed the case. The fragmentation (both with and without conversion of MB) of post-DGC was reduced by 10–20% (Table II). However, several studies report no differences in DNA damage in native and DGC populations (Stevanato et al., 2008; Thomson et al., 2009). As isolation of superior subpopulations has been shown to give higher ART success rates, so a clinical test for this population is needed. Density centrifugation isolates sperm with not only good functional parameters (WHO, 1999) but also better quality nuclear and mitochondrial DNA (Donnelly et al., 2000). Surprisingly, the SCSA appears only to be a useful prognostic tool for native semen (Larson et al., 2000; Bungum et al., 2008). Using the Comet assay to determine sperm DNA damage extends its usefulness to DGC populations as well as increasing its sensitivity.

Two recent systematic reviews have shown that the impact of sperm DNA damage on ART outcomes decreases from intrauterine insemination to IVF and is least useful in ICSI (Collins et al., 2008; Zini and Sigman, 2009), whereas in IVF, using TUNEL and SCSA assays, the OR is 1.57 (95% CI: 1.18–2.07; $P < 0.05$). In our study, using DNA strand breaks only, an OR of 4.52 (1.79–11.92) in the native semen and 6.20 (1.74–26.30) in the DGC sperm for CP following IVF indicates its promise as a prognostic test. Owing to the high levels of damage observed when both strand breaks and MB were measured, it was not possible to establish thresholds for this combined test. The OR for CP following ISCI is 1.97 (0.81–4.77) in the native semen and 2.08 (0.93–4.68) in the DGC sperm showing less robustness and supporting the combined OR of 1.14 from other studies reported by Collins et al. (2008) and Zini and Sigman (2009). This supports the belief that ICSI bypasses genetic, as well as functional defects, but is difficult to explain. The conclusion is even more surprising given that all ICSI studies in the current literature exclude the patients' poorest samples; the ∼10% that are oligoasthenoteratozoospermic as they have no sperm surplus to their clinical requirements. This creates a bias since this group has the benefit of assisted penetration through the ISCI procedure but with relatively normal semen profiles. Perhaps the successful sperm is not typical of the cohorts analysed. If it is, the wisdom of using sperm with damaged DNA is questionable even if it does result in pregnancies, given the many animal studies showing adverse effects of DNA damage on long-term health of offspring (reviewed by Aitken *et al.*, 2008; Fernadez-Gonzalez *et al.*, 2008). In conclusion, this study adds to the amassing wealth of literature by showing the usefulness of sperm DNA testing in diagnosis of male infertility and that DF (or potential DF) can predict ART outcome.

Funding

We gratefully acknowledge Hamilton Thorne Biosciences for funding L.S. in his doctoral studies.

References

Agarwal A, Said TM. Role of sperm chromatin abnormalities and DNA damage in male infertility. *Hum Reprod Update* 2003;**9**:331–345.

Agarwal A, Saleh RA, Bedaiwy MA. Role of reactive oxygen species in the pathophysiology of human reproduction. *Fertil Steril* 2003;**79**:829–843.

Agarwal A, Said TM, Bedaiwy MA, Banerjee J, Alvarez JG. Oxidative stress in an assisted reproductive techniques setting. *Fertil Steril* 2006; **86**:503–512.

Ahmadi A, Ng SC. Developmental capacity of damaged spermatozoa. *Hum Reprod* 1999;**14**:2279–2285.

Aitken RJ, Baker MA. Reactive oxygen species generation by human spermatozoa: a continuing enigma. *Int J Androl* 2002;**25**:191–194.

Aitken RJ, Baker MA. Oxidative stress, sperm survival and fertility control. *Mol Cell Endocrinol* 2006;**250**:66–69.

Aitken RJ, de Iuliis GND. Value of DNA integrity assays for fertility evaluation. *Soc Reprod Fertil Suppl* 2007;**65**:81–92.

Aitken RJ, de Iuliis GN, McLachlan RI. Biological and clinical significance of DNA damage in the male germ line. *Int J Androl* 2008;**32**:46–56.

Andersen AN, Erb K. Register data on assisted reproductive technology (ART) in Europe. Including a detailed description of ART in Denmark. *Int J Androl* 2006;**29**:12–16.

Andersen AN, Goossens V, Ferraretti S, Bhattacharya R, Felberbaum R, de Mouzon J, Nygren KG. Assisted reproductive technology in Europe, 2004. Results generated from European registers by ESHRE. *Hum Reprod* 2008;**23**:756–771.

Bakos HW, Thompson JG, Feil D, Lane M. Sperm DNA damage is associated with assisted reproductive technology pregnancy. *Int J Androl* 2008;**31**:518–526.

Barratt CLR, Aitken RJ, Bjorndahl L, Carrell DT, de Boer P, Kvist U, Lewis SEM, Perreault SD, Perry MJ, Ramos L *et al.* Sperm DNA: organization, protection and vulnerability: from basic science to clinical applications—a position report. *Hum Reprod* 2010;**25**:824–838.

Basatemur E, Sutcliffe A. Follow-up of children born after ART. *Placenta* 2008;**29**:135–140.

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

Benchaib M, Lornage J, Mazoyer C, Lejeune H, Salle B, Guerin JF. Sperm deoxyribonucleic acid fragmentation as a prognostic indicator of assisted reproductive technology outcome. *Fertil Steril* 2007;**87**:93–101.

Boe-Hansen GB, Fedder J, Ersboll AK, Christensen P. The sperm chromatin structure assay as a diagnostic tool in the human fertility clinic. *Hum Reprod* 2006;**21**:1576–1582.

Boiteux S, O'Connor TR, Lederer F, Gouyette A, Laval J. Homogeneous *Escherichia coli* FPG protein. A DNA glycosylase which excises imidazole ring-opened purines and nicks DNA at apurinic/apyrimidinic sites. *J Biol Chem* 1990;**265**:3916–3922.

Borini A, Tarozzi N, Bizzaro D, Bonu MA, Fava L, Flamigni C, Coticchio G. Sperm DNA fragmentation: paternal effect on early post-implantation embryo development in ART. *Hum Reprod* 2006;**21**:2876–2881.

Braude P, Bolton V, Moore S. Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature* 1988;**332**:459–461.

Bungum M, Humaidan P, Spano M, Jepson K, Bungum L, Giwercman A. The predictive value of sperm chromatin structure assay (SCSA) parameters for the outcome of intrauterine insemination, IVF and ICSI. *Hum Reprod* 2004;**19**:1401–1408.

Bungum M, Humaidan P, Axmon A, Spano M, Bungum L, Erenpreiss J, Giwercman A. Sperm DNA integrity assessment in prediction of assisted reproduction technology outcome. *Hum Reprod* 2007;**22**:174–179.

Bungum M, Spano M, Humaidan P, Eleuteri P, Rescia M, Giwercman A. Sperm chromatin structure assay parameters measured after density gradient centrifugation are not predictive for the outcome of ART. *Hum Reprod* 2008;**23**:4–10.

Collins AR. The comet assay for DNA damage and repair. *Mol Biotechnol* 2004;**26**:249–261.

Collins JA, Barnhart KT, Schlegel PN. Do sperm DNA integrity tests predict pregnancy with in vitro fertilization? *Fertil Steril* 2008; **89**:823–831.

Commission of the European Communities. *Commission Staff Working Document. Europe's Demographic Future: Facts and Figures*, 2009. http://ec.europa.eu/employment_social/spsi/demo_and_social_situation_en.htm.

Cooke MS, Evans MD, Dizdaroglu M, Lunec J. Oxidative DNA damage: mechanisms, mutation, and disease. *FASEB J* 2003;**17**:1195–1214.

Croteau DL, Bohr VA. Repair of oxidative damage to nuclear and mitochondrial DNA in mammalian cells. *J Biol Chem* 1997;**272**:25409–25412.

Dalzell LH, Thompson-Cree MEM, McClure N, Traub AI, Lewis SEM. Effects of 24-hour incubation after freeze-thawing on DNA fragmentation of testicular sperm from infertile and fertile men. *Fertil Steril* 2003;**79**:1670–1672.

Derijck A, van der Heijden G, Giele M, Philippens M, de Boer P. DNA double-strand break repair in parental chromatin of mouse zygotes, the first cell cycle as an origin of de novo mutation. *Hum Mol Genet* 2008;**17**:1922–1937.

Donnelly ET, Lewis SEM, McNally JA, Thompson W. In vitro fertilization and pregnancy rates: the influence of sperm motility and morphology on IVF outcome. *Fertil Steril* 1998;**70**:305–314.

Donnelly ET, McClure N, Lewis SE. The effect of ascorbate and alpha-tocopherol supplementation in vitro on DNA integrity and hydrogen peroxide-induced DNA damage in human spermatozoa. *Mutagenesis* 1999;**14**:505–512.

Donnelly ET, O'Connell M, McClure N, Lewis SEM. Differences in nuclear DNA fragmentation and mitochondrial integrity of semen and DGC human spermatozoa. *Hum Reprod* 2000;**15**:1552–1561.

Donnelly ET, Steele EK, McClure N, Lewis SEM. Assessment of DNA integrity and morphology of ejaculated spermatozoa from fertile and infertile men before and after cryopreservation. *Hum Reprod* 2001; **16**:1191–1199.

Duran EH, Morshedi M, Taylor S, Oehninger S. Sperm DNA quality predicts intrauterine insemination outcome: a prospective cohort study. *Hum Reprod* 2002;**17**:3122–3128.

European Parliament. *European Parliament Resolution of 21 February 2008 on the Demographic Future of Europe (2007/2156 (INI))*, 2008. http://www.europarl.europa.eu/sides/getDoc.do?type=TA&reference=P6-TA-2008-0066&language=EN.

Evenson DP, Jost LK, Marshall D, Zinaman MJ, Clegg E, Purvis K, de-Angelis P, Claussen OP. Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. *Hum Reprod* 1999;**14**:1039–1049.

Evenson DP, Larson KL, Jost LK. Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with the other techniques. *J Androl* 2002;**23**: 25–43.

Evenson DP, Kasperson K, Wixon RL. Analysis of sperm DNA fragmentation using flow cytometer and other techniques. *Soc Reprod Fertil Suppl* 2007;**65**:93–113.

Fairbairn DW, Olive PL, O' Neill KL. The alkaline comet assay: a comprehensive review. *Mutat Res* 1995;**339**:37–59.

Fernadez-Gonzalez R, Moreira PN, Perez-Crespo M, Sanchez-Martin M, Ramirez MA, Pericuesta E, Bilbao A, Bermejo-Alvarez P, de Dios Hourcade J, de Fonseca FR *et al.* Long-term effects of mouse intracytoplasmic sperm injection with DNA-fragmented sperm on health and behavior of adult off-spring. *Biol Reprod* 2008;**78**:761–772.

Fleming S, Green S, Hall J. Analysis and alleviation of male infertility. *Microsc Analysis* 1995;**35**:37–39.

Floyd RA. Tine role off 8-hydroxyguanine to carcinogenesis. *Carcinogenesis* 1990;**11**:1447–1450.

Fraga CG, Motchnik PA, Shigenaga MK, Helbock HJ, Jacob RA, Ames BN. Ascorbic acid protects against endogenous oxidative damage in human sperm. *Proc Natl Acad Sci USA* 1991;**88**:11003–11006.

Frydman N, Prisant N, Hesters L, Frydman R, Tachdjian G, Cohen-Bacrie P, Fanchin R. Adequate ovarian follicular status does not prevent the decrease in pregnancy rates associated with high sperm DNA fragmentation. *Fertil Steril* 2008;**89**:93–98.

Gandini L, Lombardo F, Paoli D, Caruso F, Eleuteri P, Leter G, Ciriminna R, Culasso F, Dondero F, Lenzi A *et al.* Full-term pregnancies achieved with ICSI despite high levels of sperm chromatin damage. *Hum Reprod* 2004;**19**:1409–1417.

Garrido N, Meseguer M, Simon C, Pellicer A, Remohi J. Pro-oxidative and anti-oxidative imbalance in human semen and its relation with male fertility. *Asian J Androl* 2004;**6**:59–65.

Gerrard ER, Sandlow JI, Oster RA, Burns JR, Box LC, Kolettis PN. Effect of female partner age on pregnancy rates after vasectomy reversal. *Fertil Steril* 2007;**87**:1340–1344.

Gosden R, Trasler J, Lucifero D, Faddy M. Rare congenital disorders, imprinted genes, and assisted reproductive technology. *Lancet* 2003; **361**:1975–1977.

Greco E, Scarselli F, Iacobelli M, Rienzi L, Ubaldi F, Ferrero S, Franco G, Anniballo N, Mendoza C, Tesarik J. Efficient treatment of infertility due to sperm DNA damage by ICSI with testicular spermatozoa. *Hum Reprod* 2005;**20**:226–230.

Harrison RA. Sperm evaluation: what should we be testing? In *The 6th MAFF International Workshop on Genetic Resources. Genetic Diversity and Conservation of animal Genetic Resources.* , Ibaraki, Japan, 1998.

Henkel R, Kierspel E, Hajimohammad M, Stalf T, Hoogendijk C, Mehnert C, Menkveld R, Schill WB, Kruger TF. DNA fragmentation of spermatozoa and assisted reproduction technology. *Reprod Biomed Online* 2003;**7**:477–484.

Henkel R, Hajimohammad M, Stalf T, Hoogendijk C, Mehnert C, Menkveld R, Gips H, Schill WB, Kruger TF. Influence of deoxyribonucleic acid damage on fertilization and pregnancy. *Fertil Steril* 2004;**81**:965–972.

Horak S, Polanska J, Widlak P. High levels of bulky DNA adducts in human sperm correlate with impaired fertility. *Acta Biochim Pol* 2003a; **50**:197–203.

Horak S, Polanska J, Widlak P. Bulky DNA adducts in human sperm: relationship with fertility, semen quality, smoking, and environmental factors. *Mutat Res* 2003b;**537**:53–65.

Horak S, Olejek A, Widlak P. Sperm DNA adducts impair fertilization during ICSI but not during IVF. *Folia Histochem Cytobiol* 2007;**45**:99–104.

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

Huang CC, Lin DPC, Tsao HM, Cheng TC, Liu CH, Lee MS. Sperm DNA fragmentation negatively correlates with velocity and fertilization rates but might not affect pregnancy rates. *Fertil Steril* 2005;**84**:130–140.

Hughes CM, Lewis SEM, McKelvey-Martin VJ, Thompson W. A comparison of baseline and induced DNA damage in human spermatozoa from fertile and infertile men, using a modified comet assay. *Mol Hum Reprod* 1996;**2**:613–619.

Hughes CM, Lewis SEM, McKelvey-Martin V, Thompson W. Reproducibility of human sperm DNA measurements using a single cell gel electrophoresis assay. *Mutat Res* 1997;**374**:261–268.

Hull MG, Glazener CM, Kelly NJ, Conway DI, Foster PA, Hinton RA, Coulson C, Lambert PA, Watt EM, Desai KM. Population study of causes, treatment, and outcome of infertility. *Br Med J* 1985; **291**:1693–1697.

Human Fertilization and Embryology Authority. *Human Fertilization and Embryology Authority Guide to Infertility and Directory of Clinics*, 2003. www.hfea.gov.uk/932.html.

Human Fertilization and Embryology Authority. *Human Fertilization and Embryology Authority Guide to Infertility and Directory of Clinics*, 2007. http://www.hfea.gov.uk/ivf-figures-2006.html#1280.

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

Jansen J, Olsen AK, Wiger R, Naegeli H, de Boer P, van Der HF, Holme JA, Brunborg G, Mullenders L. Nucleotide excision repair in rat male germ cells: low level of repair in intact cells contrasts with high dual incision activity in vitro. *Nucleic Acids Res* 2001;**29**:1791–1800.

Ji BT, Shu XO, Linet MS, Zheng W, Wacholder S, Gao YT, Ying DM Jin F. Paternal cigarette smoking and the risk of childhood cancer among offsprings of non smoking mothers. *J Natl Cancer Inst* 1997; **89**:238–244.

Katari S, Turan N, Bibikova M, Erinle O, Chalian R, Foster M, Gaughan JP, Coutifaris C, Sapienza C. DNA methylation and gene expression differences in children conceived in vitro or in vivo. *Hum Mol Genet* 2009;**18**:3769–3778.

Kodama H, Yamaguchi R, Fukuda J, Kasai H, Tanaka T. Increased oxidative deoxyribonucleic acid damage in the spermatozoa of infertile male patients. *Fertil Steril* 1997;**68**:519–524.

Kuznetsov SV, Sidorkina OM, Jurado J, Bazin M, Tauc P, Brochon J, Laval J, Santus R. Effect of single mutations on the structural dynamics of a DNA repair enzyme, the *Escherichia coli* formamidopyrimidine-DNA glycosylase: a fluorescence study using tryptophan residues as reporter groups. *Eur J Biochem* 1998;**253**:413–420.

Land JA, Evers JLH. Risks and complications in assisted reproduction techniques: Report of an ESHRE consensus meeting. *Hum Reprod* 2003;**18**:455–457.

Larson KL, de Jonge CJ, Barnes AM, Jost LK, Evenson DP. Relationship of assisted reproductive technique (ART) outcomes with sperm chromatin integrity and maturity as measured by the sperm chromatin structure assay (SCSA). *Hum Reprod* 2000;**15**:1717–1722.

Larson-Cook KL, Brannian JD, Hansen KA, Kasperson KM, Aamold ET, Evenson DP. Relationship between the outcomes of assisted reproductive techniques and sperm DNA fragmentation as measured by the sperm chromatin structure assay. *Fertil Steril* 2003;**80**:895–902.

Leroy T, van Hummelen P, Anard D, Castelain P, Kirsch-Volders M, Lauwerys R, Lison D. Evaluation of three methods for the detection of DNA single-strand breaks in human lymphocytes: alkaline elution, nick translation and single-cell gel electrophoresis. *J Toxicol Environ Health* 1996;**47**:409–422.

Lewis S. Is sperm evaluation useful in predicting human fertility? *Reproduction* 2007;**134**:1–11.

Lewis SEM, Aitken RJ. DNA damage to spermatozoa has impacts on fertilization and pregnancy. *Cell Tissue Res* 2005;**322**:33–41.

Lewis SEM, Boyle PM, McKinney KA, Thompson W. Total antioxidant capacity of seminal plasma is different in fertile and infertile men. *Fertil Steril* 1995;**64**:868–870.

Lewis SEM, O'Connell M, Stevenson M, Thompson-Cree L, McClure N. An algorithm to predict pregnancy in assisted reproduction. *Hum Reprod* 2004;**19**:1385–1394.

Lie RT, Lyngstadaas A, Orstavik KH, Bakketeig LS, Jacobsen G, Tanbo T. Birth defects in children conceived by ICSI compared with children conceived by other IVF-methods; a meta-analysis. *Int J Epidemiol* 2005; **34**:696–701.

Lin HH, Lee RK, Li SH, Lu CH, Sun FJ, Hwu YM. Sperm chromatin structure assay parameters are not related to fertilization rates, embryo quality, and pregnancy rates in in vitro fertilization and intracytoplasmic sperm injection, but might be related to spontaneous abortion rates. *Fertil Steril* 2008;**90**:352–359.

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

Ludwig AK, Sutcliffe AG, Diedrich K, Ludwig M. Post-neonatal health and development of children born after assisted reproduction: A systematic review of controlled studies. *Eur J Obstet Gynecol Reprod Biol* 2006; **127**:2–25.

Maccheroni C. *European Papers on the New Welfare: Implications of Demographic Change in Enlarged EU on Patterns of Saving and Consumption and in Related Consumer's Behaviour*, 2007. http://www.ec.europa.eu/employment_social/spsi/docs/social_situation/walter_consumption_summary_en.pdf (April 2008, last date accessed).

Martins CF, Dode MN, Bao SN, Rumpf R. The use of the acridine orange test and the TUNEL assay to assess the integrity of freeze-dried bovine spermatozoa DNA. *Genet Mol Res* 2007;**6**:94–104.

Morrell JM, Moffatt O, Sakkas D, Manicardi GC, Bizzaro D, Tomlinson M, Nilsson H, Holmes PV. Reduced senescence and retained nuclear DNA integrity in human spermatozoa prepared by density gradient centrifugation. *J Assist Reprod Genet* 2004;**21**:217–222.

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

Muriel L, Garrido N, Fernández JL, Remohí J, Pellicer A, de los Santos MJ, Meseguer M. Value of the sperm DNA fragmentation level, measured by the sperm chromatin dispersion (SCD) test, in the IVF and ICSI outcome. *Fertil Steril* 2006;**85**:371–383.

Nagy ZP, Liu J, Joris H, Verheyen G, Tournaye H, Camus M, Derde MC, Devroey P, van Steirteghem AC. The result of intracytoplasmic sperm injection is not related to any of the three basic sperm parameters. *Hum Reprod* 1995;**10**:1123–1129.

Nagy ZP, Verheyen G, Tournaye H, van Steirteghem AC. Special applications of intracytoplasmic sperm injection: the influence of sperm count, motility, morphology, source and sperm antibody on the outcome of ICSI. *Hum Reprod* 1998;**13**:143–154.

Ni ZY, Liu YQ, Shen HM, Chia SE, Ong CN. Does the increase of 8-hydroxydeoxyguanosine lead to poor sperm quality? *Mutat Res* 1997;**381**:77–82.

Nygren KG, Andersen AN. Assisted reproductive technology in Europe 1997. Results generated from European registers by ESHRE. *Hum Reprod* 2001;**16**:284–291.

Oehninger S. Clinical and laboratory management of male infertility: an opinion on its current status. *J Androl* 2000;**21**:814–821.

Olive PL, Johnston PJ, Banath JP, Durand RE. The alkaline comet assay: a new method to examine heterogeneity associated with solid tumours. *Nat Med* 1998;**4**:103–105.

Olsen AK, Duale N, Bjoras M, Larsen CT, Wiger R, Holme JA, Seeberg EC, Brunborg G. Limited repair of 8-hydroxy-7,8-dihydroguanine residues in human testicular cells. *Nucleic Acids Res* 2003;**31**:1351–1363.

Ozmen B, Koutlaki N, Youssry M, Diedrich K, Al-Hasani S. DNA damage of human spermatozoa in assisted reproduction: origins, diagnosis, impacts and safety. *Reprod Biomed Online* 2007;**14**:384–395.

Payne JF, Raburn DJ, Couchman GM, Price TM, Jamison MG, Walmer DK. Redefining the relationship between sperm deoxyribonucleic acid fragmentation as measured by the sperm chromatin structure assay and outcomes of assisted reproductive techniques. *Fertil Steril* 2005; **84**:356–364.

RAND. *European Union Statistical Commission*, 2006. http://www.rand.org.

Sakkas D, Alvarez JG. Sperm DNA fragmentation: mechanisms of origin, impact on reproductive outcome, and analysis. *Fertil Steril* 2010; **93**(4):1027–1036.

Sakkas D, Urner F, Bianchi P, Bizzaro D, Wagner I, Jaquenoud N, Manicardi C, Campana A. Sperm chromatin anomalies can influence decondensation after intracytoplasmic sperm injection. *Hum Reprod* 1996;**11**:837–843.

Saleh RA, Agarwal A, Nada EA, El-Tonsy MH, Sharma RK, Meyer A, Nelson DR, Thomas AJ. Negative effects of increased sperm DNA damage in relation to seminal oxidative stress in men with idiopathic and male factor infertility. *Fertil Steril* 2003;**79**:1597–1606.

Scott L. Pronuclear scoring as a predictor of embryo development. *Reprod Biomed Online* 2003;**6**:201–214.

Seli E, Gardner DK, Schoolcraft WB, Moffatt O, Sakkas D. Extent of nuclear DNA damage in ejaculated spermatozoa impacts on blastocyst development after *in vitro* fertilization. *Fertil Steril* 2004; **82**:378–383.

Shen HM, Chia SE, Ong CN. Evaluation of oxidative DNA damage in human sperm and its association with male infertility. *J Androl* 1999; **20**:718–723.

Sies H. Strategies of antioxidant defence. *Eur J Biochem* 1993; **215**:213–219.

Sies H, Stahl W, Sundquist AR. Antioxidant function of vitamins. *Ann N Y Acad Sci* 1992;**669**:7–20.

Silber SJ, Nagy Z, Devroey P, Camus M, van Steirteghan A. The effect of female age and ovarian reserve on pregnancy rate in male infertility: treatment of azoospermia with sperm retrieval and intractyoplasmic sperm injection. *Hum Reprod* 1997;**12**:2693–2700.

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

Sorahan T, Lancashire RJ, Hulten MA, Peck I, Stewart AM. Childhood cancer and parental use of tobacco: deaths from 1953 to 1955. *Br J Cancer* 1997;**75**:134–138.

Spano M, Bonde JP, Hjullund HI, Kolstad HA, Cordelli E, Leter G, The Danish First Pregnancy Planner Study Team. Sperm chromatin damage impairs human fertility. *Fertil Steril* 2000;**73**:43–50.

Stevanato J, Bertolla RP, Barradas V, Spaine DM, Cedenho AP, Ortiz V. Semen processing by density gradient centrifugation does not improve sperm apoptotic deoxyribonucleic acid fragmentation rates. *Fertil Steril* 2008;**90**:889–890.

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

Sutcliffe AG, Ludwig M. Outcome of assisted reproduction. *Lancet* 2007; **370**:351–359.

Terriou P, Giorgetti C, Hans E, Salzmann J, Charles O, Cignetti L, Avon C, Roulier R. Relationship between even early cleavage and day 2 embryo score and assessment of their predictive value for pregnancy. *Reprod Biomed Online* 2007;**14**:294–299.

Tesarik J, Greco E, Mendoza C. Late, but not early, paternal effect on human embryo development is related to sperm DNA fragmentation. *Hum Reprod* 2004;**19**:611–615.

Thomson LK, Fleming SD, Aitken RJ, de Iuliis GN, Zieschang JA, Clark AM. Cryopreservation-induced human sperm DNA damage is predominantly mediated by oxidative stress rather than apoptosis. *Hum Reprod* 2009;**24**:2061–2070.

Tice RR, Andrews PW, Singh NP. The single cell gel assay: a sensitive technique for evaluating intercellular differences in DNA damage and repair. *Basic Life Sci* 1990;**53**:291–301.

Tomlinson MJ, Kessopoulou E, Barratt CL. The diagnostic and prognostic value of traditional semen parameters. *J Androl* 1999;**20**:588–593.

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

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

van den Bergh M, Hohl MK, de Geyter CH, Stalberg AM, Limoni C. Ten years of Swiss National IVF Register FIVNAT-CH. Are we making progress? *Reprod Biomed Online* 2006;**11**:632–640.

van Royen E, Mangelschots K, Vercruyssen M, de Neubourg D, Valkenburg M, Rychaert G, Gerris J. Multinucleation in cleavage stage embryos. *Hum Reprod* 2003;**18**:1062–1069.

van Steirteghem A, Liu J, Joris H, Nagy Z, Jannssenswillen C, Tournaye H. Assisted fertilisation by subzonal insemination and intracytoplasmic sperm injection. *Hum Reprod* 1993;**8**:1061–1066.

Virro MR, Larson-Cook KL, Evenson DP. Sperm chromatin structure assay (SCSA) parameters are related to fertilization, blastocyst development, and ongoing pregnancy in *in vitro* fertilization and intracytoplasmic sperm injection cycles. *Fertil Steril* 2004;**81**:1289–1295.

Williams C, Sutcliffe A. Infant outcomes of assisted reproduction. *Early Hum Dev* 2009;**85**:673–677.

Woldringh GH, Besselink DE, Tillema AHJ, Hendriks JCM, Kremer JAM. Karyotyping, congenital anomalies and follow-up of children after intracytoplasmic sperm injection with non-ejaculated sperm: a systematic review. *Hum Reprod Update* 2010;**16**:12–19.

World Health Organization. *WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction*, 3rd edn. Cambridge, UK: Cambridge University Press, 1992.

World Health Organization. *WHO Laboratory Manual for the Examination of Human Semen and Sperm-cervical Mucus Interaction*, 4th edn. Cambridge, UK: Cambridge University Press, 1999.

Xu DX, Shen HM, Zhu QX, Chua L, Wang QN, Chia SE, Ong CN. The associations among semen quality, oxidative DNA damage in human spermatozoa and concentrations of cadmium, lead and selenium in seminal plasma. *Mutat Res* 2003;**534**:155–163.

Zenzes MT, Puy LA, Bielecki R, Reed TE. Detection of benzo[a]pyrene diol epoxide-DNA adducts in embryos from smoking couples: evidence for transmission by spermatozoa. *Mol Hum Reprod* 1999; **5**:125–131.

Ziebe S, Devroey P. Assisted reproduction technologies are an integrated pare of national strategies addressing denographic and reproductive challenges. *Hum Reprod Update* 2008;**14**:583–592.

Zini A, Sigman M. Are Tests of sperm DNA damage clinically useful? Pros and cons. *J Androl* 2009;**30**:219–229.

Zini A, Meriano J, Kader K, Jarvi K, Laskin CA, Cadesky K. Potential adverse effect of sperm DNA damage on embryo quality after ICSI. *Hum Reprod* 2005;**20**:3476–3480.

Zini A, Boman JM, Belzile E, Ciampi A. Sperm DNA damage is associated with an increased risk of pregnancy loss after IVF and ICSI: systematic review and meta-analysis. *Hum Reprod* 2008;**23**:2663–2668.