

ORIGINAL ARTICLE

Sperm DNA damage is associated with assisted reproductive technology pregnancy

Hassan W. Bakos,*† Jeremy G. Thompson,* Deanne Feil*† and Michelle Lane*†

*Research Centre for Reproductive Health, Discipline of Obstetrics and Gynaecology, School of Paediatrics and Reproductive Health, University of Adelaide, Adelaide, SA, Australia, and †Repromed, Dulwich, SA, Australia

Keywords:

pregnancy, semen analysis, single embryo transfer, sperm DNA damage

Correspondence:

Michelle Lane, Research Centre for Reproductive Health, Discipline of Obstetrics and Gynaecology, School of Paediatrics and Reproductive Health, University of Adelaide, Level 2, Medical School South, Frome Road, Adelaide, SA, 5005, Australia. E-mail: michelle.lane@adelaide.edu.au

Received 19 February 2007; revised 19 April 2007; accepted 15 May 2007

doi:10.1111/j.1365-2605.2007.00803.x

Summary

The literature suggests an association between sperm DNA damage and assisted reproductive technology (ART) outcomes. However, previous studies involved the transfer of multiple embryos, which has complicated the interpretation of the results. The aim of this study was to determine the relationship between the levels of sperm DNA damage and fertilization rate, embryo development as well as pregnancy outcome, following single embryo transfer. Patients ($n = 113$) undergoing in vitro fertilization (IVF) ($n = 45$) and intra-cytoplasmic sperm injection (ICSI) ($n = 68$) were assessed for their levels of sperm DNA damage in the sample used for insemination. DNA damage was determined using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-nick end labelling (TUNEL). The relationship between DNA damage and outcomes were assessed using regression analysis. Overall data showed no association between sperm DNA damage and fertilization rate, or embryo development in vitro. However, when IVF was the insemination method, there was a significant negative correlation between fertilization rates and sperm DNA damage ($p < 0.05$). When ICSI was the insemination technique, low sperm DNA damage was associated with successful pregnancy ($37.8 \pm 5.7\%$ DNA damaged sperm) compared with failed implantation ($52.9 \pm 3.9\%$ DNA damaged sperm, $p < 0.05$). Our results suggest that sperm DNA damage as measured by the TUNEL assay may provide an indicator for patients with poor fertilization rates and/or those unable to achieve pregnancy following ART treatment.

Introduction

Male infertility continues to be a significant issue constituting approximately 50% of infertility cases (Lamb & Lipshultz, 2000), with defective sperm function being the most common defined cause (Hull *et al.*, 1985), affecting about one in 20 men in Australia (McLachlan & de Kretser, 2001). While it has been long established that perturbations in conventional sperm parameters such as concentration, motility and morphology are associated with male infertility (Edizione, 1993), these parameters are not predictive of the integrity of the sperm genome. Furthermore, the use of intra-cytoplasmic sperm injection (ICSI) in assisted reproductive technology (ART) has made these conventional parameters redundant for sperm selection, as only one spermatozoon for each oocyte inseminated is required to achieve fertilization.

In the general population, elevated levels of sperm DNA damage have been associated with early pregnancy loss (Carrell *et al.*, 2003). Furthermore, it has been hypothesized that environmental factors such as cigarette smoke may induce DNA damage in the spermatozoa and thereby increase the risk of childhood cancer in the offspring (Lewis & Aitken, 2005). Therefore, the integrity of the paternal genome appears important for embryonic and foetal development and also long-term health of the offspring. For ART, studies have also shown that increased sperm DNA damage is associated with decreased fertilization rates (Sun *et al.*, 1997; Lopes *et al.*, 1998), embryo cleavage rates (Sun *et al.*, 1997; Tomsu *et al.*, 2002; Saleh *et al.*, 2003) and clinical pregnancy rates (Larson *et al.*, 2000; Tomsu *et al.*, 2002; Saleh *et al.*, 2003; Virro *et al.*, 2004; Greco *et al.*, 2005c). However, there is considerable contradiction in the

literature with several other studies demonstrating that these same parameters are not affected by increased levels of sperm DNA damage (Larson *et al.*, 2000; Morris *et al.*, 2002; Virro *et al.*, 2004). The discrepancies seen in the literature may be due to several reasons (i) whether the analysis was performed on the raw semen sample or the washed sample for in vitro fertilization, (ii) lack of distinction between the use of IVF or ICSI for insemination, (iii) the different methods used to measure sperm DNA damage and (iv) the transfer of multiple embryos to patients.

In the current study, the aim was to determine if DNA damage was a predictor of fertilization rates, embryo cleavage rates, embryo grade in vitro, as well as pregnancy following single embryo transfer. Secondly, the differences between IVF and ICSI in relation to sperm DNA damage were elucidated.

Materials and methods

Sample collection

All patients included in this study attended the infertility clinic, Repromed, Dulwich, South Australia, between April 2005 and August 2006. A total of 113 samples were collected from couples undergoing insemination by either IVF ($n = 45$) or ICSI ($n = 68$). Sperm DNA damage was assessed on the surplus washed sperm used for the IVF or ICSI treatment. In addition, a retrospective chart review was performed to obtain the patients' semen analysis prior to their treatment and semen analysis on the day of the cycle. Both paternal age and maternal age were also obtained. Ethical approval was granted from the Women's and Children's Human Ethics Committee.

Conventional semen analysis

Evaluation of sperm samples both for the initial diagnosis prior to the treatment as well as for semen analysis on the day of treatment were performed according to the World Health Organization criteria (Edizione, 1993).

Sperm isolation using density gradient separation

Motile sperm were separated from semen samples using 40 and 80% Puresperm gradients (Nicadon Laboratories AB, Gothenburg, Sweden). Semen was layered onto the gradient and centrifuged at 100 *g* for 20 min. The pellet was removed and washed once in medium G-Sperm (Series III; Vitrolife AB, Gothenburg, Sweden) and re-suspended in medium G-FERT (Series III; Vitrolife AB). All sperm was stored for a minimum of 3 h at 37 °C in 6% CO₂ in air before use.

Assessment of sperm DNA damage

All assessments of sperm DNA integrity were performed blinded to the outcomes of the laboratory procedures and were performed by the same individual throughout the study.

Spermatozoa were smeared on polylysine-coated slides (Menzel-Glaser, Braunschweig, Germany), air-dried and fixed with 4% paraformaldehyde in phosphate-buffered solution (PBS; JRH Biosciences, Lenexa, KS, USA) for 1 h at room temperature. Smears were then maintained in PBS overnight at 4 °C. The following morning the smears were permeabilized with 0.5% Triton X-100 in 0.1% sodium citrate for 1 h. Smears were then washed twice with PBS and incubated with the terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL). The assay was performed using a Cell Detection Kit (Roche, Mannheim, Germany) for 1 h at 37 °C. Smears were then washed twice with PBS and stained with propidium iodide (PI, 1 mg/mL) to identify sperm nuclei. Smears were then washed with PBS twice and a drop of a pre-prepared mixture of gold antifade reagent (Molecular Probes, Eugene, OR, USA) and 99% Glycerol (Sigma Chemical Co., New South Wales, Australia) applied. Slides were examined using fluorescence microscopy. Two individual filters were utilized to capture the nuclear signal (PI; excitation 540–565 nm, emission 605–660 nm) and the TUNEL signal (FITC; excitation 465–495 nm, emission 515–555 nm). The two captured images were superimposed using IPLab software (version 3.61; Scanalytics, Inc., Rockville, MD, USA). The percentage of sperm DNA damage was calculated as the number of TUNEL positive sperm from the total number of sperm nuclei. At least 200 sperm were counted for each sample.

For a positive control, permeabilized sperm were incubated with 3 IU DNase (Sigma Chemical Co.) at 37 °C prior to the incubation with the TUNEL mixture. For a negative control, the terminal transferase was omitted from the reaction.

Ovarian stimulation

All female partners in this study underwent controlled ovarian stimulation by standard midluteal phase GnRH agonist (Synarel; Serono, Frenchs Forest, New South Wales, Australia) down regulation, followed by stimulation with recombinant FSH (Gonal-F, Serono) for 11–15 days as described previously (Schoolcraft *et al.*, 1999). Stimulation was monitored by ultrasound and serum oestradiol levels and an injection of hCG (Pregnyl, Serono) was given when one-two follicles had a mean diameter of >18 mm. Oocyte retrieval was scheduled 36 h later (Schoolcraft *et al.*, 1999).

Fertilization assessment, embryo culture, cleavage and grading

All oocytes collected were either inseminated using conventional IVF or ICSI as per Schoolcraft *et al.* (1999). Insemination occurred between 4 and 6 h after oocyte collection. For conventional IVF, oocytes were placed in 50 μL drops of fertilization medium G-FERT (Series III; Vitrolife AB) with 30 000 spermatozoa. For ICSI, a single motile spermatozoon was selected and injected into the oocyte. Fertilization was assessed the following morning (16–18 h post-insemination) by the presence of two pronuclei and two polar bodies (2PN). All 2PN's were cultured in groups of two to four in G1 medium (Series III; Vitrolife AB). At 25 h (± 30 min) post-insemination, cleavage to the two-cell stage was assessed and those embryos at the two-cell stage were designated as early cleavage-stage embryos. The following morning (40–42 h post-insemination), embryo morphology was assessed, based on embryo cell number and the degree of fragmentation and assigned a grade of 1 (best quality)–4 (poor quality). All decisions for which embryos to transfer were based on morphology. Good quality embryos were defined as the ones receiving scores of either 1 or 2 as these are considered to be suitable for freezing.

Embryo transfer and pregnancy determination

Embryos were transferred in 10 μL of EmbryoGlue medium (Vitrolife) using a Sydney IVF Transfer Catheter (Cook Australia, Queensland, Australia) under ultrasound guidance. Biochemical pregnancy was determined by the presence of serum hCG of >20 IU on day 14 following embryo transfer, followed by an 8-week scan for presence of a foetal heartbeat.

Statistical analysis

SPSS statistical package (SPSS Inc., version 13.0; SPSS Inc., Chicago, IL, USA) was used to analyse all data. Linear regression analysis was performed to determine any correlation between conventional semen analysis, age and the percentage of sperm DNA damage. For all analyses involving cycle outcomes, maternal age was fitted as a covariate. Using the Spearman rank correlation coefficient linear regression analysis was performed to correlate the percentage of sperm DNA damage and fertilization rate, early embryo cleavers and good quality embryos. Univariate generalized linear modelling was performed to determine if different thresholds of sperm DNA damage had an effect on fertilization rate, early cleavage-stage rate, embryo quality and pregnancy outcome with Bonferoni's

multiple comparison procedure used to assess for differences between individual treatments.

Results

Cohort characteristics

For the couples ($n = 113$) involved in this study, mean paternal age was 37.8 ± 0.5 , mean maternal age was 36.1 ± 0.8 . The mean number of oocytes collected was 9.5 ± 0.6 , the mean number of oocytes fertilized normally was, 5.6 ± 0.4 , and the mean number of early cleaver embryos to the two-cell was 1.7 ± 0.2 .

Sperm DNA damage, conventional sperm analysis and paternal age

The clinical decision for insemination treatment was based on the conventional sperm parameters of sperm concentration, motility and morphology performed by an accredited pathology laboratory. There was no difference in the percentage of sperm DNA damage in the insemination sample in patients that underwent IVF ($39.4 \pm 2.7\%$) insemination compared with ICSI ($45.9 \pm 2.7\%$).

The percentage of TUNEL positive sperm in the inseminated samples were not correlated with conventional sperm assessment parameters used routinely for determination of sperm quality. Using the Spearman rank correlation coefficient on all samples, no correlation was found between the percentage of sperm with DNA damage and the sperm concentration (Fig. 1A), motility of the ejaculated sperm (Fig. 1B), or percentage of normal morphology (Fig. 1C). Similarly, no correlation was found between the percentage of sperm with DNA damage and the above parameters on the day of the treatment (data not shown). Results also showed no correlation between the percentage of sperm with DNA damage and paternal age (Fig. 1D). Interestingly, there was a positive association between the period of abstinence and the percentage of TUNEL positive sperm ($p < 0.01$; Fig. 1E).

Sperm DNA damage and fertilization rate

When data for both insemination procedures (IVF and ICSI) were combined, there was no correlation between the percentage of sperm with DNA damage and fertilization rate (Fig. 2A). However, a significant negative correlation was found between the percentage of sperm with DNA damage and fertilization rate in patients undergoing IVF insemination ($p < 0.05$; Fig. 2B). In contrast, when ICSI was used as the insemination method, there was no correlation between percentage of sperm with DNA

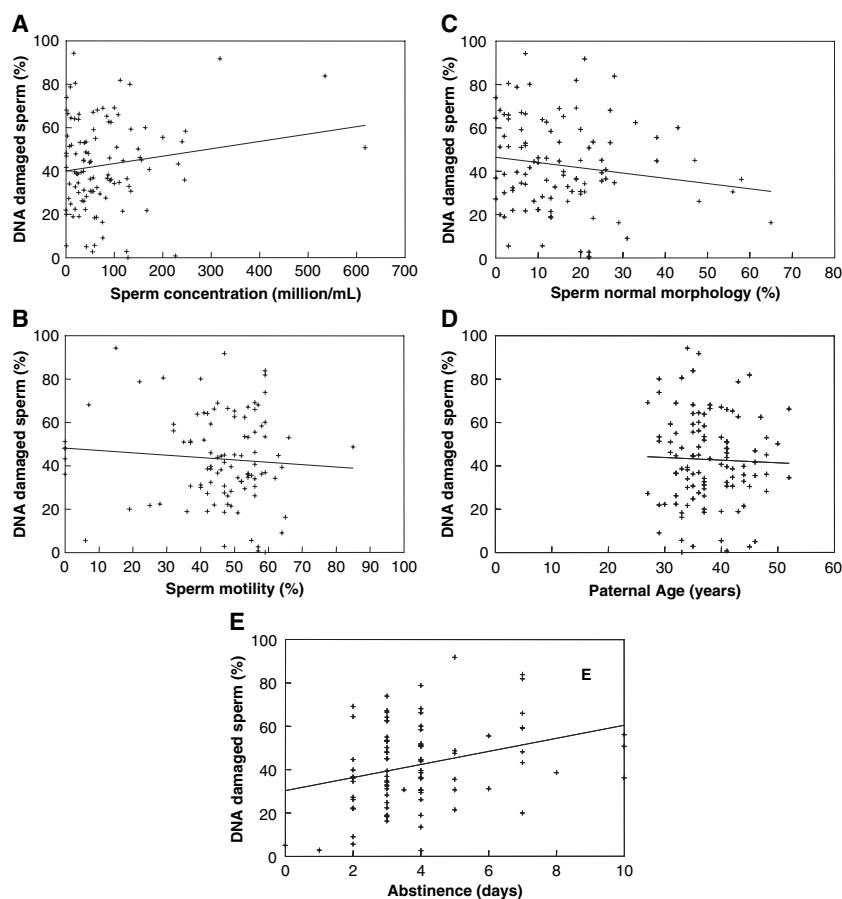


Figure 1 Correlation of sperm DNA damage with (A): sperm concentration (B): sperm motility, (C): sperm morphology (D): paternal age and (E): abstinence. No significant correlations were found between the percentage of sperm DNA damage and concentration, motility, morphology or paternal age. A correlation was found between sperm DNA damage and abstinence ($p < 0.01$).

damage and fertilization rate (Fig. 2C). Furthermore, when the data were subdivided into three groups based on the percentage of DNA damage: Low: $\leq 35\%$; Moderate: 35–55% and High $>55\%$, there was a significant increase in the fertilization rate after IVF in patients who had low TUNEL positive sperm compared with those in the high group ($p < 0.05$; Fig. 3A). Dividing the patients into the three DNA damage thresholds had no effect on ICSI fertilization rate (Fig. 3B).

Sperm DNA damage and embryo development

Assessment of whether sperm DNA damage influenced the quality of the embryos was assessed by determining the correlation of sperm DNA damage with the percentage of early cleavage embryos on day 1 (data not shown), and embryo morphology on day 2 (Figs 4 and 5). A weak correlation was found between the percentage of sperm DNA damage and the incidence of early cleavage and also embryo grade in all patients. However, this was accounted for by maternal age when modelled as a covariate. No significant differences were detected between embryo quality parameters and the percentage of TUNEL

positive sperm when the data were divided according to the insemination technique (Figs 4B and C). Similarly, when the data were subdivided into three groups according to the percentage of DNA damage, there were no significant differences in rate of early cleavage (data not shown) or embryo grade irrespective of the insemination procedure (Figs 5A and B).

Sperm DNA damage and pregnancy outcomes

The overall pregnancy rate of the patients in this study was 39.7% following single embryo transfer. The percentage of sperm DNA damage following single embryo transfer was significantly lower in those who achieved a pregnancy ($34 \pm 3.4\%$) compared with those who did not ($48 \pm 3.8\%$; $p < 0.01$). The effect of DNA damage in the sperm sample on pregnancy outcome was most evident in the ICSI group ($p < 0.05$; Fig. 6). There was a trend for an effect of DNA damage on IVF pregnancy outcome, however, the effect was less pronounced (Fig. 6). Interestingly, there were three blighted ovum pregnancies reported, all which had a DNA damage percentage $>50\%$ (mean: 57.9%).

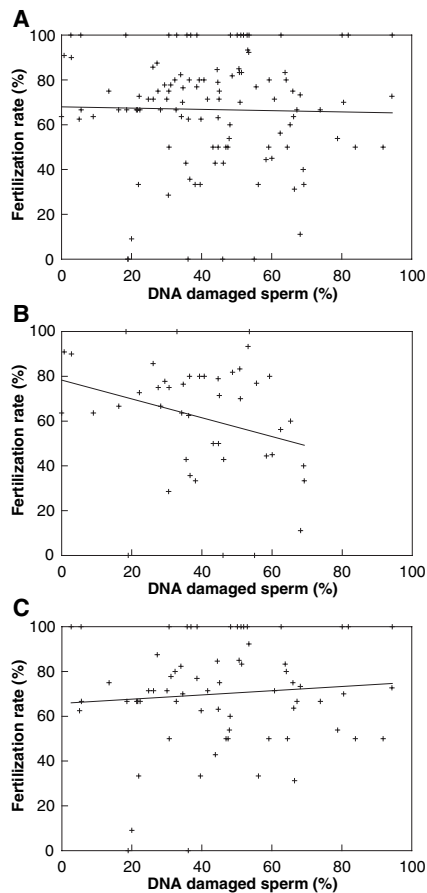


Figure 2 Correlation of sperm DNA damage with fertilization rate following (A): in vitro fertilization (IVF) and intra-cytoplasmic sperm injection (ICSI) inseminations combined, (B): IVF insemination only and (C): ICSI insemination only. (A) and (C): showed no significant correlation between sperm DNA damage and fertilization rate. (B): showed a significant negative correlation between sperm DNA damage and fertilization rate ($p < 0.05$).

Discussion

In this study we demonstrated for the first time that achieving pregnancy with IVF/ICSI is negatively associated with sperm DNA damage in single embryo transfers. Previously studies had transferred different numbers of embryos within groups of patients making it difficult to establish a direct effect on implantation potential in isolation from differences in fertilization rates and embryo selection (Morris *et al.*, 2002; Virro *et al.*, 2004).

Sperm DNA damage is now measured predominantly by three different procedures: (i) TUNEL (direct measure of the presence of endogenous nicks in both single and double DNA strands), (ii) Comet (an electrophoresis assay, which evaluates how well the DNA is packaged within the nucleus) or (iii) sperm chromatin structure

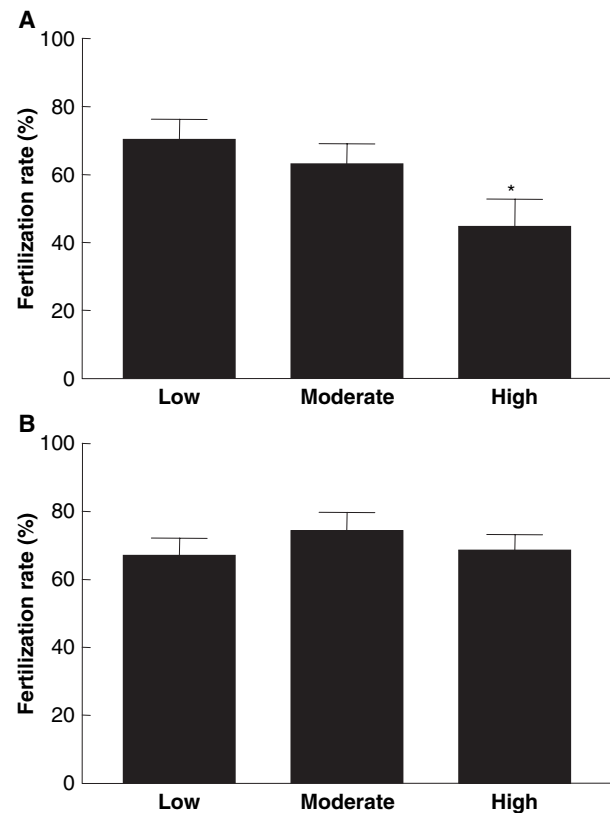


Figure 3 Effect of sperm DNA damage on fertilization rate. Data were divided into three groups based on the percentage of DNA damage: Low: $<35\%$; Moderate: $35\text{--}55\%$; and High $>55\%$. (A): in vitro fertilization insemination, (B): intra-cytoplasmic sperm injection insemination. Data expressed as mean \pm SEM. * significantly different from the low damage group ($p < 0.05$).

assay (SCSA; is an assessment of sperm chromatin integrity by measuring the susceptibility of DNA to acid or heat-induced denaturation). Our study has determined that the assessment of the DNA damage using TUNEL in the sperm sample used for insemination was useful in predicting fertilization rate after IVF insemination. However, more significantly we have determined that there is a significant reduction in pregnancy rate after ICSI in couples where the sperm sample used for insemination had high rates of DNA damage. Interestingly, this was not the case for IVF patients. Therefore, we have demonstrated that the presence of DNA damage in the sperm appears to reduce the ability to fertilize normally. Bypassing this with ICSI enables fertilization; however, the resultant embryo is significantly less competent to undergo implantation. Therefore, our data raises the question as to whether ICSI is the best treatment option for patients with high percentage of DNA damage.

The results of the present study demonstrated that using the TUNEL technique, there was a significant

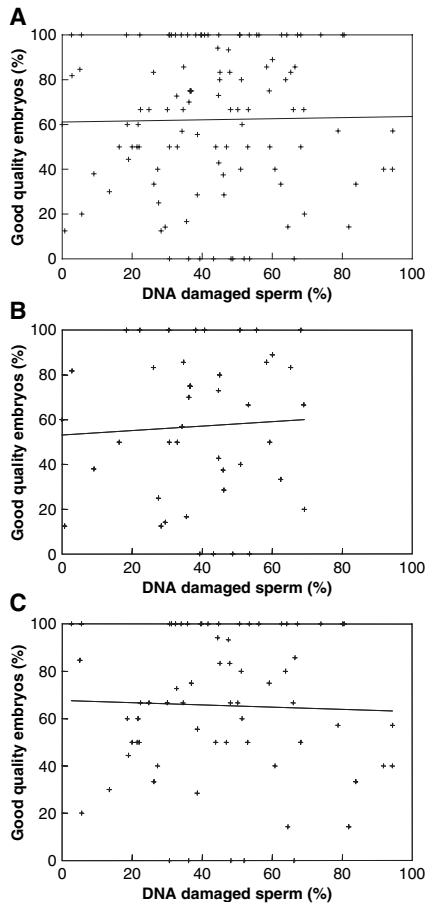


Figure 4 Correlation of sperm DNA damage with good quality embryos following (A): in vitro fertilization (IVF) and intra-cytoplasmic sperm injection (ICSI) inseminations combined, (B): IVF insemination only and (C): ICSI insemination only. No correlation between sperm DNA damage and the percentage of good quality embryos was observed.

negative correlation between increased sperm DNA damage and fertilization rate after IVF insemination. This concurs with other studies, which also measured sperm DNA damage on IVF patients using the TUNEL technique (Sun *et al.*, 1997; Benchaib *et al.*, 2003). Interestingly, this is contrary to studies which used the Comet and SCSA assays, which measure susceptibility to DNA damage (Larson *et al.*, 2000; Morris *et al.*, 2002; Tomsu *et al.*, 2002). The apparent discrepancy in these studies may indicate a difference in the ability of the measurement techniques in predicting fertilization outcomes. It may also be a result of differences between the assessments of the raw semen sample or the washed sample for insemination. It would appear from our data and that of others that for fertilization outcome, TUNEL, which is a direct measure of DNA damage, may be more predictive of fertilization outcomes than those techniques that meas-

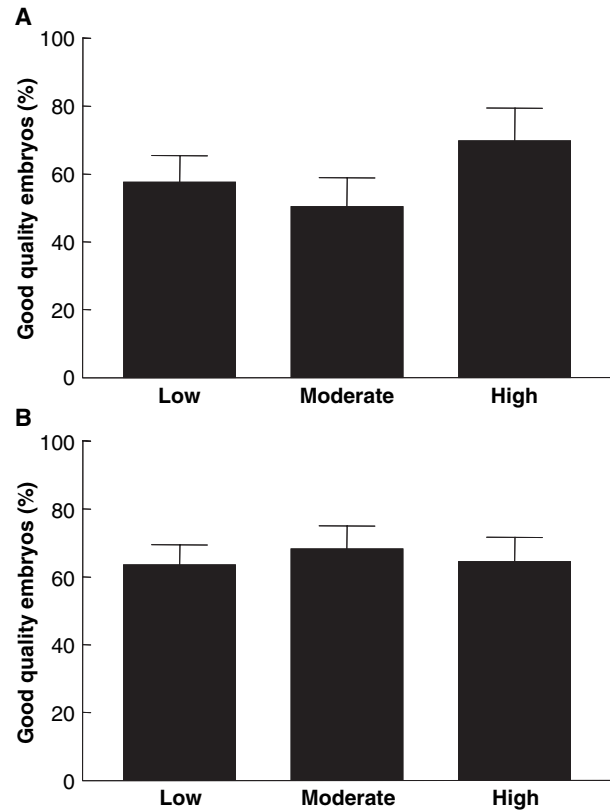


Figure 5 Effect of DNA damage in sperm sample on embryo quality. Data were divided into three groups based the percentage of DNA damage: Low: <35%; Moderate: 35–55% and High >55%. (A): in vitro fertilization insemination, (B): intra-cytoplasmic sperm injection insemination. Data expressed as mean \pm SEM. There was no effect of sperm DNA damage on embryo quality.

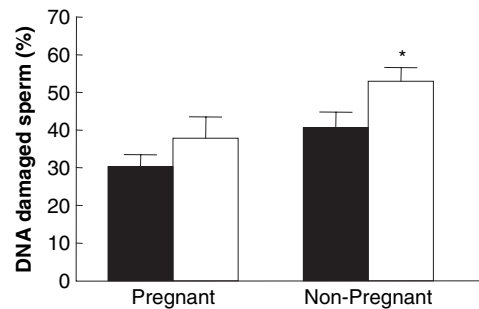


Figure 6 Effect of sperm DNA damage on pregnancy outcome. Solid bars represent in vitro fertilization insemination. Open bars represent intra-cytoplasmic sperm injection insemination. Data expressed as mean \pm SEM. *Significantly different from the non-pregnant group ($p < 0.05$).

ure susceptibility to damage. In contrast to the outcome after insemination by IVF, our data showed no correlation between percentage of sperm DNA damage and

fertilization rate after ICSI. Previous studies (Twigg *et al.*, 1998; Greco *et al.*, 2005b) have shown a similar result, while others (Lopes *et al.*, 1998; Benchaib *et al.*, 2003) have shown a correlation. Therefore, the question of whether sperm DNA damage is associated with ICSI fertilization rates remains unclear.

The difference in fertilization efficiency after IVF or ICSI in samples with high sperm DNA damage suggests that intact DNA may be necessary for certain processes such as the acrosome reaction and sperm capacitation or the ability of the sperm to penetrate the zona pellucida and fuse with the vitelline membrane of the oocyte (Lewis & Aitken, 2005). These steps are necessary in order for successful fertilization to occur in both natural conception and IVF; and are clearly bypassed in ICSI, where the sperm is physically deposited into the ooplasm.

Our data showed no association between the percentage of sperm DNA damage and the number of early cleavage-stage embryos or embryo grade regardless of the insemination technique. This is consistent with other studies (Sun *et al.*, 1997; Benchaib *et al.*, 2003; Greco *et al.*, 2005c). A single study did show some association however, maternal age was not fitted as a covariate in the analysis and therefore may have confounded the result (Sun *et al.*, 1997). This observation that sperm DNA damage did not influence embryo quality is perhaps not surprising considering that the majority of embryos in the current study were transferred on days 2 or 3, and it is generally accepted that the paternal genome is activated at the four- to eight-cell stage in humans (Artley *et al.*, 1992).

Current sperm selection criteria for ICSI patients are based on morphology and motility. Moreover, our data showed no association between sperm DNA damage and sperm motility or morphology. Therefore, it is very likely that in sperm samples with high percentage of TUNEL positive sperm, the ICSI operator is frequently selecting sperm with DNA damage to inject into the oocyte. The fact that our ICSI data showed a strong association between increased sperm DNA damage and implantation failure despite successful fertilization and embryo development is intriguing. This suggests that while sperm with DNA damage have the ability to fertilize an oocyte and proceed through the early stages of embryogenesis (days 2–3) following ICSI, once the paternal genome is activated, failure of blastocyst formation and/or failure of implantation occurs. This is presumed to be due to abortive paternal transcription of damaged genes (Ainsworth *et al.*, 2005) or epigenetic effects (Braude *et al.*, 1988). It is unclear from our data whether these late paternal effects (Tesarik *et al.*, 2004) could influence blastocyst development as most of our embryos were transferred on days 2 or 3. However, blastocyst development has been assessed in relation to sperm DNA damage in a previous

study (Seli *et al.*, 2004). Interestingly, Seli *et al.* (2004) showed that when the cut off score for sperm DNA damage (as measured by TUNEL) was >50% four of eight patients had no blastocysts develop in vitro. This is compared with only two of the 41 remaining patients, who all had sperm DNA damage of ≤50%. This evidence illustrates the importance of DNA integrity and its association with the embryo's ability to undergo post-fertilization paternal genome activation. Our data in relation to pregnancy support Seli's findings where couples who were not able to achieve a pregnancy had significantly higher sperm DNA damage compared with those who did. In fact, the average sperm DNA damage in our study for those who did not achieve pregnancy was 48% and only two pregnancies were achieved above this threshold. Interestingly, the three blighted ovum pregnancies in our study were to couples where the male partner had a mean sperm DNA damage percentage of 57.9%. This further illustrates the importance of intact sperm DNA to achieve successful implantation and raises the question whether sperm DNA damage has any effect on trophoblast to inner cell mass ratio; as a blighted ovum pregnancy lacks presence of a foetal pole, which is derived from the inner cell mass. This has yet to be determined in any model system. Therefore, our data overall suggests that the spermatozoon used for oocyte injection in ICSI patients may have DNA damage. This in turn may affect the ability of the embryo to develop to the blastocyst stage or undergo successful implantation.

It has been reported that oocytes may be able to repair DNA damage in sperm. However, it is unclear as to how the oocyte exactly deals with sperm DNA damage. Most evidence has been indirect using repair inhibitors such as arabinofuranosyl cytosine (ara-C), 3-aminobenzamide (3AB) and caffeine (Matsuda & Tobari, 1989). Using these inhibitors it was concluded that DNA damage in sperm is repairable in the fertilized eggs (Matsuda & Tobari, 1989). Furthermore, it has been found that while DNA damaged mouse and human sperm had the ability to fertilize an oocyte, development to the blastocyst stage was inhibited beyond a certain level of DNA damage (Ahmadi & Ng, 1999). Therefore, there may be a threshold of damage that can be repaired by the oocyte or embryo (Shimura *et al.*, 2002).

The subject of DNA damage is important in fertility. Therefore, new methods to isolate populations of sperm that are relatively free of DNA damage are required, such as the new electrophoretic technique proposed by Ainsworth *et al.* (2005, 2007). This technique was shown to isolate a subpopulation of sperm with reduced sperm DNA damage as measured by TUNEL, compared with current density separation methods. Furthermore it is clearly desirable in a clinical environment to treat the

issue of DNA damage in the first place and potentially avoid the need for intervention in conception. Therefore, antioxidant therapies such the one proposed in a recent study (Greco *et al.*, 2005a) should be considered. Greco *et al.* (2005a) showed that a combination of vitamin C and E taken orally had the ability to reduce sperm DNA damage in a placebo-controlled trial. It may also be worth suggesting to patients who have high levels of DNA damage to limit their abstinence period before an ART cycle. Our data suggests that longer periods of abstinence are associated with higher sperm DNA damage. This strategy may reduce the levels of damage sperm and improve outcomes. A previous study determined that retrieving testicular samples selects sperm with lower levels of damage suggesting that the majority of damage occurs in the epididymis (Greco *et al.*, 2005c). Our data showing increased damage with increased abstinence would support this notion.

In conclusion, the percentage of sperm DNA damage as measured by the TUNEL assay was useful in identifying patients who were more susceptible to an unsuccessful ART cycle. Pregnancy rates in ICSI patients were significantly lower when the sperm sample used for insemination had increased DNA damage. Therefore, TUNEL would be a useful test to incorporate into the assessment of patients for ART treatments especially where there is a history of failed implantations.

Acknowledgements

HWB was the recipient of a Queen Elizabeth Hospital Honours Research Scholarship, JGT is the recipient of an NHMRC Senior Research Fellowship, DF is the recipient of an NHMRC Biomedical Postgraduate Scholarship and ML is the recipient of a RD Wright Career Development award from the NHMRC. This work was supported by a grant awarded to ML from the NHMRC. The authors would like to thank Mr David Froiland for his assistance with the TUNEL technique and all of the clinical and scientific staff at Repromed for their support.

References

- Ahmadi, A. & Ng, S. C. (1999) Fertilizing ability of DNA-damaged spermatozoa. *Journal of Experimental Zoology* 284, 696–704.
- Ainsworth, C., Nixon, B. & Aitken, R. J. (2005) Development of a novel electrophoretic system for the isolation of human spermatozoa. *Human Reproduction* 20, 2261–2270.
- Ainsworth, C., Nixon, B., Jansen, R. P. & Aitken, R. J. (2007) First recorded pregnancy and normal birth after icsi using electrophoretically isolated spermatozoa. *Human Reproduction* 22, 197–200.
- Artley, J. K., Braude, P. R. & Johnson, M. H. (1992) Gene activity and cleavage arrest in human pre-embryos. *Human Reproduction* 7, 1014–1021.
- Benchaib, M., Braun, V., Lornage, J., Hadj, S., Salle, B., Lejeune, H. & Guerin, J. F. (2003) Sperm DNA fragmentation decreases the pregnancy rate in an assisted reproductive technique. *Human Reproduction* 18, 1023–1028.
- Braude, P., Bolton, V. & Moore, S. (1988) Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature* 332, 459–461.
- Carrell, D. T., Liu, L., Peterson, C. M., Jones, K. P., Hatasaka, H. H., Erickson, L. & Campbell, B. (2003) Sperm DNA fragmentation is increased in couples with unexplained recurrent pregnancy loss. *Archives of Andrology* 49, 49–55.
- Edizione, T. (1993) WHO laboratory manual for the examination of human seminal fluid and the interaction of sperm with cervical mucus. *Annali dell Istituto Superiore di Sanita* 29(Suppl. 2), 1–98.
- Greco, E., Iacobelli, M., Rienzi, L., Ubaldi, F., Ferrero, S. & Tesarik, J. (2005a) Reduction of the incidence of sperm DNA fragmentation by oral antioxidant treatment. *Journal of Andrology* 26, 349–353.
- Greco, E., Romano, S., Iacobelli, M., Ferrero, S., Baroni, E., Minasi, M. G., Ubaldi, F., Rienzi, L. & Tesarik, J. (2005b) Icsi in cases of sperm DNA damage: Beneficial effect of oral antioxidant treatment. *Human Reproduction* 20, 2590–2594.
- Greco, E., Scarselli, F., Iacobelli, M., Rienzi, L., Ubaldi, F., Ferrero, S., Franco, G., Anniballo, N., Mendoza, C. & Tesarik, J. (2005c) Efficient treatment of infertility due to sperm DNA damage by icsi with testicular spermatozoa. *Human Reproduction* 20, 226–230.
- Hull, M. G., Glazener, C. M., Kelly, N. J., Conway, D. I., Foster, P. A., Hinton, R. A., Coulson, C., Lambert, P. A., Watt, E. M. & Desai, K. M. (1985) Population study of causes, treatment, and outcome of infertility. *Br Med J (Clin Res Ed)* 291, 1693–1697.
- Lamb, D. J. & Lipshultz, L. I. (2000) Male infertility: recent advances and a look towards the future. *Curr Opin Urol* 10, 359–362.
- Larson, K. L., DeJonge, C. J., Barnes, A. M., Jost, L. K. & Evenson, D. P. (2000) Sperm chromatin structure assay parameters as predictors of failed pregnancy following assisted reproductive techniques. *Human Reproduction* 15, 1717–1722.
- Lewis, S. E. & Aitken, R. J. (2005) DNA damage to spermatozoa has impacts on fertilization and pregnancy. *Cell and Tissue Research* 322, 33–41.
- Lopes, S., Sun, J. G., Jurisicova, A., Meriano, J. & Casper, R. F. (1998) Sperm deoxyribonucleic acid fragmentation is increased in poor-quality semen samples and correlates with failed fertilization in intracytoplasmic sperm injection. *Fertility and Sterility* 69, 528–532.
- Matsuda, Y. & Tobari, I. (1989) Repair capacity of fertilized mouse eggs for X-ray damage induced in sperm and mature oocytes. *Mutation Research* 210, 35–47.

- McLachlan, R. I. & de Kretser, D. M. (2001) Male infertility: the case for continued research. *Medical Journal of Australia* 174, 116–117.
- Morris, I. D., Illott, S., Dixon, L. & Brison, D. R. (2002) The spectrum of DNA damage in human sperm assessed by single cell gel electrophoresis (comet assay) and its relationship to fertilization and embryo development. *Human Reproduction* 17, 990–998.
- Saleh, R. A., Agarwal, A., Nada, E. A., El-Tonsy, M. H., Sharma, R. K., Meyer, A., Nelson, D. R. & Thomas, A. J. (2003) Negative effects of increased sperm DNA damage in relation to seminal oxidative stress in men with idiopathic and male factor infertility. *Fertility and Sterility* 79(Suppl. 3), 1597–1605.
- Schoolcraft, W. B., Gardner, D. K., Lane, M., Schlenker, T., Hamilton, F. & Meldrum, D. R. (1999) Blastocyst culture and transfer: Analysis of results and parameters affecting outcome in two in vitro fertilization programs. *Fertility and Sterility* 72, 604–609.
- Seli, E., Gardner, D. K., Schoolcraft, W. B., Moffatt, O. & Sakkas, D. (2004) Extent of nuclear DNA damage in ejaculated spermatozoa impacts on blastocyst development after in vitro fertilization. *Fertility and Sterility* 82, 378–383.
- Shimura, T., Inoue, M., Taga, M., Shiraishi, K., Uematsu, N., Takei, N., Yuan, Z. M., Shinohara, T. & Niwa, O. (2002) P53-dependent s-phase damage checkpoint and pronuclear cross talk in mouse zygotes with x-irradiated sperm. *Molecular and Cellular Biology* 22, 2220–2228.
- Sun, J. G., Jurisicova, A. & Casper, R. F. (1997) Detection of deoxyribonucleic acid fragmentation in human sperm: correlation with fertilization in vitro. *Biology of Reproduction* 56, 602–607.
- Tesarik, J., Greco, E. & Mendoza, C. (2004) Late, but not early, paternal effect on human embryo development is related to sperm DNA fragmentation. *Human Reproduction* 19, 611–615.
- Tomsu, M., Sharma, V. & Miller, D. (2002) Embryo quality and ivf treatment outcomes may correlate with different sperm comet assay parameters. *Human Reproduction* 17, 1856–1862.
- Twigg, J. P., Irvine, D. S. & Aitken, R. J. (1998) Oxidative damage to DNA in human spermatozoa does not preclude pronucleus formation at intracytoplasmic sperm injection. *Human Reproduction* 13, 1864–1871.
- Virro, M. R., Larson-Cook, K. L. & Evenson, D. P. (2004) Sperm chromatin structure assay (scca) parameters are related to fertilization, blastocyst development, and ongoing pregnancy in in vitro fertilization and intracytoplasmic sperm injection cycles. *Fertility and Sterility* 81, 1289–1295.