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Insulin dependant diabetes mellitus: implications for male reproductive function

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BACKGROUND: Diabetes mellitus (DM) is increasing in men of reproductive age. Despite this, the prevalence of diabetes in men attending fertility clinics is largely unknown. Furthermore, studies examining the effects of DM on sperm fertility potential have been limited to conventional semen analysis. METHODS: Conventional semen analysis (semen volume, sperm count, motility and morphology) was performed for 27 diabetic (mean age 34 $\pm$ 2 years) and 29 non-diabetic subjects (control group, men undergoing routine infertility investigations, mean age 33 $\pm$ 1 years). Nuclear DNA (nDNA) fragmentation was assessed using the alkaline Comet assay and mitochondrial DNA (mtDNA) deletions by Long-PCR. RESULTS: Other than a small, but significant, reduction in semen volume in diabetic men (2.6 versus 3.3 ml; $P < 0.05$), conventional semen parameters did not differ significantly from control subjects. Diabetic subjects had significantly higher mean nDNA fragmentation (53 versus 32%; $P < 0.0001$) and median number of mtDNA deletions (4 versus 3; $P < 0.05$) compared with control subjects. CONCLUSIONS: Diabetes is associated with increased sperm nuclear and mtDNA damage that may impair the reproductive capability of these men.

Keywords: diabetes mellitus; sperm; DNA damage; male infertility; Comet assay

Introduction

Diabetes mellitus (DM) represents one of the greatest threats to modern global health. Its incidence is rising rapidly. In the year 2000, the World Health Organization (WHO) reported that 177 million people were affected by diabetes worldwide but by 2025, this figure is projected to rise to over 300 million (WHO, 2002). Factors such as obesity, population growth and ageing are thought to be largely responsible (Wild *et al.*, 2004).

The vast majority (>90%) of patients with type-1 diabetes are diagnosed before the age of 30 (Williams and Pickup, 2004). This type of diabetes is rising by 3% per annum in European children, with an increasing number being diagnosed in early childhood (EURODIAB, 2000). Over the next 10 years, this will result in a 50% increase in prevalence (Silink, 2002). As a consequence diabetes will affect many more men prior to and during their reproductive years.

DM may affect male reproductive function at multiple levels as a result of its effects on the endocrine control of spermatogenesis, spermatogenesis itself or by impairing penile erection and ejaculation (Sexton and Jarow, 1997). There are a number of reports in the literature examining the effects of diabetes on the endocrine control of spermatogenesis (Daubresse *et al.*, 1978; Handelsman *et al.*, 1985; Dinulovic and Radonjic, 1990; Garcia-Diez *et al.*, 1991; Baccetti *et al.*, 2002; Ballester *et al.*, 2004). However, the results of these studies have been conflicting and the reported abnormalities are unlikely to impair reproductive function significantly in isolation (Sexton and Jarow, 1997). Diabetes is, however, a well-recognized cause of male sexual dysfunction, which in itself may contribute to subfertility.

Studies of sperm quality in diabetes have been limited to light microscopic assessment of conventional semen parameters (semen volume, sperm count, motility and morphology). Conventional semen analysis is now recognized to be of limited value in the determination of fertility status (Jequier, 2005) unless there are more extreme abnormalities such as severe oligoasthenoteratozoospermia or azoospermia. The paucity of studies addressing the effects of DM on human male reproductive function and the conflicting nature of existing data have resulted in a distinct lack of consensus in the current literature as to the extent of the problem.

Data from animal models strongly suggest that DM impairs male fertility. Numerous studies have demonstrated a marked reduction in fecundity when male animals are diabetic (Frenkel *et al.*, 1978; Murray *et al.*, 1983; Cameron *et al.*,

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1990; Ballester *et al.*, 2004; Scarano *et al.*, 2006), as well as an impairment of sperm quality (Amaral *et al.*, 2006; Scarano *et al.*, 2006). If similar effects exist in the context of human male reproduction, the rising rates of diabetes may well pose a significant problem to human fertility. Despite this, the potential impact of the increase in diabetes in young men and its effect on their reproductive health has received comparatively little attention to date.

To our knowledge, at a population rather than an individual level, there has been no comparison of diabetic and non-diabetic male fecundity. However, there is evidence to suggest a higher prevalence of infertility in diabetic men (Sexton and Jarow, 1997) and an increase in adverse reproductive outcomes such as spontaneous abortion in their partners (Babbott *et al.*, 1958). In view of this, it is essential that a logical and rigorous scientific analysis of the effects of diabetes on male reproductive function be performed.

An alternative approach to the light microscopic assessment of sperm to investigate male fertility is the assessment of sperm nuclear DNA (nDNA) or mitochondrial DNA (mtDNA) quality. These have been shown to be 'proxy' indicators of male fertility status (Agarwal and Said, 2003; O'Brien and Zini, 2005; St John *et al.*, 2005). Together, an assessment of sperm nDNA fragmentation and mtDNA deletion number and size has been shown to have prognostic value in assisted reproductive outcomes (Lewis *et al.*, 2004). To our knowledge, this paper is the first to compare sperm from diabetic and non-diabetic men using conventional and molecular techniques.

Materials and Methods

Subjects

Male type-1 diabetics aged between 18 and 60, attending the Regional Centre for Endocrinology and Diabetes at the Royal Victoria Hospital, Belfast, for routine assessment of their diabetes were invited to participate in this study (mean age 34 ± 2; $n = 27$). Men attending the Queen's University of Belfast Andrology Laboratory at the Regional Fertility Centre, Royal Maternity Hospital, Belfast, for semen analysis as part of routine infertility investigations (mean age 33 ± 1; $n = 29$) were employed as a control group. Within this control group, only semen samples approximating normal WHO criteria were included in the study. A venous blood sample was taken at the time of semen analysis for the measurement of glycosylated haemaglobin (HbA1c) to assess recent glycaemic control. 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 and motility according to WHO recommendations (WHO, 1999). Sperm morphology was assessed according to Tygerberg Strict Criteria (Kruger *et al.*, 1988). Semen analysis was performed within 1 h of ejaculation, following a period of incubation at 37°C to allow for liquefaction. The remaining semen was divided into aliquots and incubated at 37°C in preparation for further analysis by Comet assay.

Aliquots of semen (containing 3–5 million sperm) from each subject were diluted in cryovials with Sperm freeze, (Fertipro N.V., 8370 Beernem, Belgium) in a ratio of 1:0.7, then plunge frozen in liquid nitrogen, following static phase vapour cooling, for a period of 15 min. DNA from these samples was subsequently extracted and used for mtDNA assessment by Long-PCR as described below.

Assessment of sperm nDNA fragmentation by modified alkaline single cell gel electrophoresis (Comet) assay

nDNA fragmentation was assessed using an alkaline single cell gel electrophoresis (Comet) assay as previously modified by our group (Hughes *et al.*, 1997; Donnelly *et al.*, 1999). Briefly, aliquots of neat semen were adjusted with PureSperm® wash (Nidacon International AB, Mölndal, Sweden) to give a sperm concentration of 6×10^6 ml^{-1}. Those semen samples with an initial concentration less than this were used without dilution. Following the initial preparation of the sperm sample, all subsequent steps were carried out in a climate controlled room (18°C) under yellow light, to prevent induced DNA damage.

Embedding of sperm in agarose gel

Fully frosted microscope slides (Surgipath Europe, Peterborough, UK) were heated gently, coated with 100 μl of 0.5% normal melting point agarose (Sigma-Aldrich, Poole, UK) in phosphate-buffered saline (Sigma), kept at 45°C and immediately covered with a glass coverslip (22 × 50 mm). Slides were left at ambient temperature (18°C) to allow the agarose to solidify. The coverslips were removed, and 10 μl of diluted semen (6×10^6 ml^{-1}) was mixed with 75 μl of 0.5% low-melting point agarose (Sigma) at 37°C. This cell suspension was pipetted over the first layer of gel, covered with a glass coverslip and allowed to solidify at ambient temperature.

Lysing of cells and decondensation of DNA

Coverslips were removed and the slides immersed in a Coplin jar containing 22.5 ml of fresh lysis solution (2.5 M NaCl, 100 mM EDTA and 10 mM Tris (pH 10), with 1% Triton X-100 (Sigma) added just prior to use), for 1 h at 4°C. Subsequently, 2.5 mLs of 0.1 M dithiothreitol [DTT] (Sigma) was added to achieve a final concentration of 10 mM for a further 30 min at 4°C. This was followed by 2.5 mLs of 40 mM lithium diiodosalicylate [LIS] (Sigma) to achieve a final concentration of 4 mM which was then incubated at ambient temperature for 90 min.

Unwinding of DNA

Slides were removed from the lysis solution and drained of any residual fluid. Fresh alkaline electrophoresis solution was prepared (300 mM NaOH, 1 mM EDTA; Sigma) and poured into a horizontal gel electrophoresis tank. The agarose coated slides were placed side by side in the tank, for 20 min, allowing the exposed DNA to unwind.

Separation of DNA fragments by electrophoresis

Electrophoresis was carried out for 10 min at 25 V, with the current adjusted to 300 mA, by the addition or removal of buffer from the tank. Following this, slides were removed from the tank, drained and flooded with three changes of neutralization buffer (0.4 M Tris; pH 7.5; Sigma), removing any residual alkali or detergents that may interfere with staining. Slides were stained with 50 μl of 20 μg/ml ethidium bromide (Sigma), covered with a glass coverslip and stored in a humidified container in darkness at 4°C overnight, until analysis.

Image analysis

Slides were viewed on a Nikon E600 epifluorescence microscope (Nikon, Tokyo, Japan) equipped with an excitation filter of 515–560 nm from a 100 W mercury lamp and a barrier filter of 590 nm.

The degree of sperm DNA fragmentation was determined using an image analysis system (Komet 3.1, Kinetic Imaging, Nottingham, UK) to analyse 50 sperm per slide (Hughes *et al.*, 1997).

Assessment of sperm mtDNA deletions by Long-PCR

Sperm DNA isolation

DNA was isolated from sperm samples using a Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN, USA). Briefly, samples previously frozen in liquid nitrogen were allowed to defrost at room temperature and then centrifuged at 16 000 *g* for 1 min to pellet cells. The supernatant was removed and 300 μl of cell lysis solution (Gentra) added and pipette mixed. Following this, 12 μl of 1 M DTT (Sigma) and 1.5 μl of 20 mg ml^{-1} Proteinase k (Sigma) were added. Samples were inverted 25 times and incubated at 55°C overnight to allow complete lysis of the cells.

After cooling to room temperature, 1.5 μl of RNAse A solution (Gentra) was added to the cell lysate and incubated for 1 h at 37°C. Samples were again allowed to cool to room temperature prior to adding 100 μl of protein precipitation solution (Gentra). Samples were placed on ice for 5 min and then centrifuged at 16 000 *g* for 4 min to pellet the precipitated proteins. DNA was precipitated by pouring the supernatant containing the DNA into an Eppendorf tube containing 300 μl of 100% isopropanol (Sigma) and inverting 50 times. Following centrifugation at 16 000 *g* for 1 min and removal of supernatant, the tube was inverted on absorbent paper to drain for 15 min. The DNA pellet was subsequently washed with 300 μl 70% ethanol (Sigma) by inversion several times before centrifugation at 16 000 *g* for 1 min. The supernatant was removed and the DNA pellet allowed to dry. Following this, DNA was re-hydrated by adding 50 μl of DNA hydration solution (Gentra) to the tube and incubating for 1 h at 65°C.

DNA quantification was performed on each sample using a nanospectrophotometer (NanoDrop® ND-1000 v 3.0.0, NanoDrop Technologies, Rockland, USA) at a wavelength of 260 nm. This was first calibrated using ultra pure water (DEPC Water, Gibco, Invitrogen, Paisley, UK). Extracted DNA was stored at 4°C prior to assessment of mtDNA.

Long-PCR amplification

Long-PCR amplification of mtDNA was performed in a 50 μl volume using Bio-X-Act DNA polymerase (Bioline, London, UK) and a Hybaid touchdown thermal cycling system (Hybaid Ltd, Middlesex, UK). Reaction mixtures contained 1 × Optiform buffer (Bioline), 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 500 ng DNA template, 2 U of Bio-X-act and 0.5 μM of each primer (D6: 5′-TCT AGA GCC CAC TGT AAA G-3′, L strand sequence, position 8286–8304 and R10: 5′-AGT GCA TAC CGC CAA AAG A-3′, L strand sequence, position 421–403) (Lestienne *et al.*, 1997). In brief, initial denaturation was performed at 94°C for 2 min, followed by 34 cycles of denaturation at 94°C for 10 s, annealing at 52°C for 30 s and extension at 68°C for 10 min. The ‘semi-hot’ technique was used, in which tubes containing all of the reaction components were placed in the thermal cycler at the beginning of the denaturation phase. Positive and negative controls were included in each set of reactions using primers for β-Actin with and without genomic DNA respectively. Long-PCR was repeated in duplicate samples to ensure reproducibility and identical deletions were found. Reaction products were separated by electrophoresis in a 0.8% agarose gel (Sigma) made with 1 × Tris–Acetate–EDTA buffer (10 × TAE Buffer; Gibco-BRL, Life Technologies, Paisley, UK), containing 1 μg ml^{-1} ethidium bromide (Sigma). A voltage of 120 V was applied for 60 min. Following electrophoresis, mtDNA deletions were visualized using an ultraviolet bio-imaging system (EC3 Imaging System, UVP Ltd, Cambridge, UK).

The number of mtDNA deletions was calculated by counting the total number of bands detected for each subject from Long-PCR products. The deletion size was calculated by comparing its position on the gel with an adjacent molecular weight ladder (HyperLadder I, Bioline). The mean deletion size was calculated by dividing the sum of all deletion sizes by the total number of deletions.

Statistical analysis

Data was analysed using the Statistical Package for the Social Sciences (SPSS 11) for Mac OS 10 (SPSS Inc., Chicago, IL, USA www.SPSS.com). Values are expressed as mean ± SEM. Semen profiles and nDNA fragmentation data from controls and diabetic men were compared using Student’s *t*-test. Sperm concentrations and total sperm output were normalized using a square root transformation. To account for the non-Gaussian distribution of the mtDNA data, the non-parametric Mann–Whitney *U*-test was used to compare median values for control and diabetic subjects.

Results

Comparison of conventional semen profiles from control and diabetic subjects

Semen samples from non-diabetic control and diabetic groups were compared (Table 1). As expected, mean HbA1c was significantly higher in the diabetic group. There was no significant difference between groups in the age of subjects. Abstinence times did not differ between the control and diabetic groups. Semen volume in diabetic men was significantly less than for non-diabetic controls ($P < 0.05$). However, no significant differences were observed in sperm concentration, total sperm output, percentage motility or percentage normal morphology. None of the diabetic or control subjects had significant leucocytospermia (WHO $>1 \times 10^6$ ml^{-1}).

nDNA fragmentation of control and diabetic sperm assessed by the alkaline Comet assay

The mean percentage of fragmented sperm nDNA as determined by the Comet assay was significantly higher in sperm from diabetic subjects ($n = 24$) compared with that from non-diabetic controls ($n = 23$) (53 ± 3 versus 32 ± 2%; $P < 0.0001$). Our group has previously reported an intra-assay coefficient of variation <6% for this assay (Hughes *et al.*, 1997).

Number and size of mtDNA deletions in control and diabetic sperm

The median number of mtDNA deletions was significantly higher in sperm from diabetic subjects ($n = 23$) when compared with controls ($n = 21$) (4 [3–6] versus 3 [1–4]; $P < 0.05$) [Fig. 1a]. None of the sperm from diabetic subjects displayed wild-type mtDNA, compared with 10% of controls. Ninety-one percent of diabetic men displayed more than two mtDNA deletions compared with 67% of controls ($P < 0.05$). The median size of mtDNA deletions did not differ significantly between the two groups (7 kb [6–7] versus 7 kb [6–7]; $P > 0.05$) [Fig. 1b].

Table 1: Comparison of age, HbA1c and semen profiles from control and diabetic men

	Group			
	Control ($n = 29$)	Diabetic ($n = 27$)	P-value	WHO[a] normal
Age (years)[b]	32.7 ± 0.7	34.0 ± 2.0	0.52	
[b]HbA1c (%)[b]	5.3 ± 0.1	8.2 ± 0.2	<0.0001	
Semen volume (ml)[b]	3.3 ± 0.2	2.6 ± 0.3	<0.05	2–4
Sperm concentration ($10^6 ml^{-1}$)[c]	51 [28–100]	64 [30–151]	0.22	>20
Total sperm output (10^6)[c]	173 [89–338]	198 [99–450]	0.84	—
Motility (%)[b]	47.3 ± 2.8	46.0 ± 4.2	0.79	>50
Normal morphology (%)[b]	11.7 ± 0.8	11.1 ± 0.6	0.56	>14

[a]World Health Organization normal reference values (WHO, 1999).
[b]Values expressed as mean ± SEM.
[c]Values expressed as median [inter-quartile range].

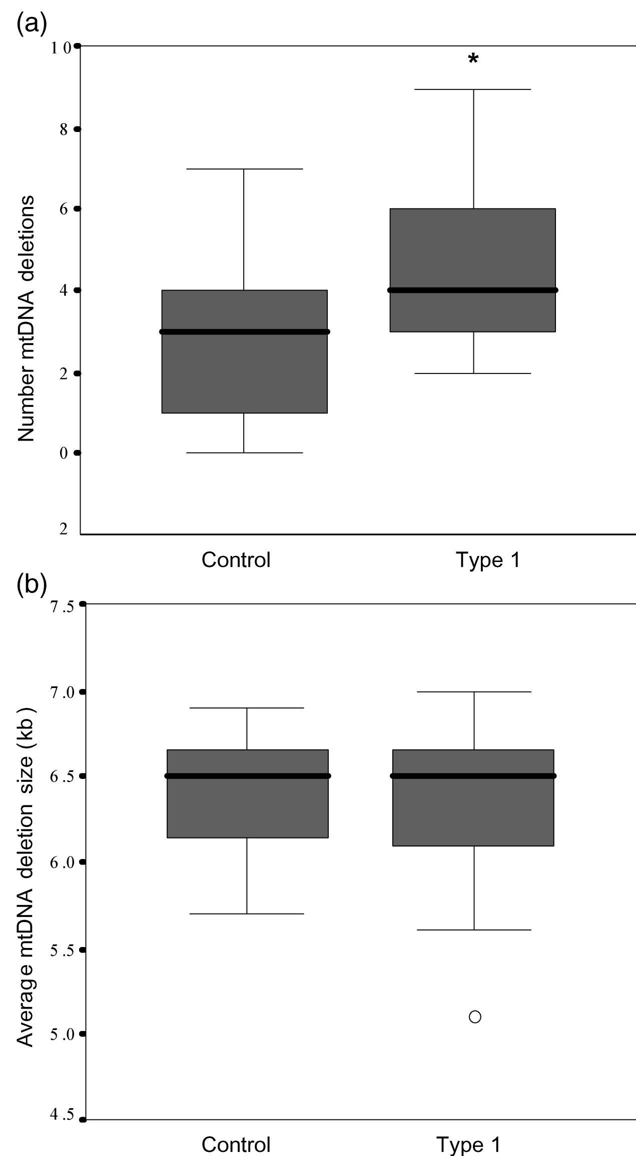

Figure 1: A boxplot comparing mtDNA deletons in sperm from control ($n = 29$) and diabetic men ($n = 27$, Type 1 diabetes mellitus) (a) mtDNA deletion number. (b) mean mtDNA deletion size. $*P < 0.05$; Mann–Whitney U-test

Discussion

The rising incidence of DM worldwide will inevitably result in an increased prevalence in men of reproductive age. Infertility is already a major health problem in both the developed and developing world with up to one in six couples requiring specialist investigation or treatment in order to conceive (Hull *et al.*, 1985; Schmidt and Munster, 1995). Disorders of sperm are thought to be either causative or contributory in 40–50% of infertile couples (Thonneau *et al.*, 1991; Sharlip *et al.*, 2002). Moreover, the last 50 years has seen an apparent decline in semen quality (Carlsen *et al.*, 1992). The increasing incidence of systemic diseases such as DM may further exacerbate this decline in male fertility.

Previous studies have estimated the prevalence of type-1 DM in subfertile men at ~1% (Greenberg *et al.*, 1978; Sexton and Jarow, 1997). On the basis of background prevalence of DM and male infertility in this age group, this figure was expected to be around 0.3% (Sexton and Jarow, 1997). Our group conducted a postal survey of UK fertility clinics (data not shown) and found that few had data regarding the prevalence of DM among male patients. This reinforces the fact that DM is not currently seen as a particularly relevant issue in the assessment of male fertility. This would suggest the need for a large-scale epidemiological study, investigating the relationship between male fertility and DM.

Animal studies using rodent models of streptozotocin-induced DM have demonstrated a reduction in sperm counts and quality (Ballester *et al.*, 2004; Amaral *et al.*, 2006; Scarano *et al.*, 2006). In addition, a marked reduction in fecundity has been observed after as little as 15 days following the injection of streptozotocin (Scarano *et al.*, 2006). Other groups have reported similar findings after longer periods of induced diabetes (2–6 months) (Frenkel *et al.*, 1978; Cameron *et al.*, 1990; Ballester *et al.*, 2004). The associated reduction in fertility is more pronounced when DM is induced in pre-pubertal animals (Frenkel *et al.*, 1978). Furthermore, spontaneously occurring DM in the BB Wistar rat, is also associated with a significant reduction in fertility (Murray *et al.*, 1983; Cameron *et al.*, 1990), thus eliminating any possible confounding effects of diabetogenic agents as a primary cause. These studies support the hypothesis that DM impairs male reproductive function.

Studies of semen quality in diabetic men have, so far, been limited to the use of conventional light microscopy. A reduction in all semen parameters (semen volume, sperm count, motility and morphology) has been observed in two studies of type-1 diabetics (Padron *et al.*, 1984; Garcia-Diez *et al.*, 1991). However, Handelsman *et al.* (1985) found only semen volume and total sperm output to be significantly lower in diabetic men. A large study of diabetic men (Ali *et al.*, 1993) showed an increase in sperm concentration and total sperm output but a concomitant reduction in motility

and no difference in sperm morphology. Vignon *et al.* (1991) demonstrated higher sperm concentrations and abnormal morphology with no difference in motility. Not surprisingly, many of these diabetic men with normal semen parameters had fathered children and the authors concluded that DM, in itself, was not a cause of subfertility. In those studies demonstrating an adverse effect of diabetes on semen parameters, poor metabolic control and associated neuropathy have been shown to be important predictors of the extent of impairment (Sexton and Jarow, 1997).

Conventional semen analysis remains core to the evaluation of male fertility in the clinical setting. However, although the WHO reference values for semen parameters are published and widely used, considerable controversy exists as to the value of recommended 'normal' thresholds (Ombelet *et al.*, 1997; Bonde *et al.*, 1998; Chia *et al.*, 1998; Guzick *et al.*, 2001). A man with an apparently normal semen analysis may still be subfertile (Bonde *et al.*, 1998; Saleh *et al.*, 2002). In addition, large intra-individual variations occur over time (Mallidis *et al.*, 1991; WHO, 1999; Alvarez *et al.*, 2003).

Although we have observed a significant reduction in mean semen volume in diabetic men, it still remains within the normal range set by the WHO (1999). In addition, we have not found significant differences in any of the other conventional semen parameters. It is our contention that the significant differences lie at a 'molecular' and not a 'cellular' level.

In view of the limitations of conventional semen analysis, we determined sperm nDNA and mtDNA status as molecular biomarkers of fertility potential. The need for the evaluation of sperm DNA quality to be introduced into the clinical setting has been acknowledged (Perreault *et al.*, 2003; Aitken, 2006). These tests of 'genetic integrity' provide additional independent information on sperm quality (Trisini *et al.*, 2004), identifying abnormalities that are not apparent in conventional semen profiles (Saleh *et al.*, 2002). However, these tests have not yet gained clinical popularity as they are laborious, time consuming and relatively expensive. In addition, useful clinical thresholds have yet to be established for many of these techniques (Perreault *et al.*, 2003).

This is the first report to our knowledge of sperm nDNA and mtDNA quality in men with diabetes. Our study identifies important evidence of increased nDNA fragmentation and mtDNA deletions in sperm from diabetic men. These findings are concerning, as they may have implications for the fertility, risk of spontaneous abortion and health of the children of diabetic men.

The relationship between genomic integrity and male fertility has been the subject of intense research over the past decade (O'Brien and Zini, 2005; Evenson and Wixon, 2006). Numerous reports have demonstrated an increase in sperm DNA damage in infertile men (Kodama *et al.*, 1997; Evenson *et al.*, 1999; Spano *et al.*, 2000; Zini *et al.*, 2001). Furthermore, sperm DNA has been shown to be predictive of the time taken to achieve a pregnancy (Loft *et al.*, 2003).

Damage to sperm DNA does not necessarily preclude fertilization (Aitken *et al.*, 1998; Ahmadi and Ng, 1999a,b). The oocyte has a limited ability to repair damaged sperm DNA (Matsuda and Tobari, 1989; Genesca *et al.*, 1992) and fragmentation beyond this threshold may result in increased rates of embryonic failure and pregnancy loss (Ahmadi and Ng, 1999a,b). In the context of spontaneous conception, sperm DNA quality has been found to be poorer in couples with a history of spontaneous abortion (Carrell *et al.*, 2003a,b).

Perhaps more worryingly, increased sperm DNA damage has been implicated in the future health of resulting offspring (Brinkworth, 2000; Aitken *et al.*, 2003a,b; Aitken, 2004). Children of men who smoke, and thus have increased levels of oxidative sperm DNA damage (Fraga *et al.*, 1996), are more likely to suffer from childhood cancers, particularly leukaemia and lymphoma (Ji *et al.*, 1997). In one series, 14% of all childhood cancers were linked to paternal smoking (Sorahan *et al.*, 1997). Thus, sperm DNA damage in men can have significant and long lasting effects, which are not simply limited to male infertility itself but perpetuated in future generations to the detriment of their offspring.

A variety of approaches exist for the assessment of sperm nDNA. We used the alkaline Comet assay, previously modified for use with sperm by this group among others (Hughes *et al.*, 1997; Donnelly *et al.*, 1999). The Comet assay is a simple, reliable and reproducible technique to measure DNA fragmentation in individual sperm (Hughes *et al.*, 1997). Various versions of this assay exist, however, the alkaline Comet assay allows for the widest detection of DNA damage (Hartmann *et al.*, 2003). Assessment of sperm DNA quality using this method has been shown to be predictive of pregnancy rates in assisted conception (Morris *et al.*, 2002; Lewis *et al.*, 2004).

Various studies have shown that fertility declines when sperm DNA fragmentation measured by the sperm chromatin structure assay (SCSA) is elevated: $> 30\%$ (Evenson *et al.*, 1999) $> 40\%$ (Spano *et al.*, 2000). In addition, the authors of a study employing Terminal dUTP nick-end labelling (TUNEL assay), showed that by using a threshold of 20% fragmentation, a specificity of 89% and sensitivity of 97% for distinguishing between fertile and infertile men could be achieved (Sergerie *et al.*, 2005). These studies, among others, reinforce the value of these tests assessing the genomic integrity of sperm in the prediction of male fertility potential (Agarwal and Allamaneni, 2005).

The aetiology of sperm DNA damage is multi-factorial (Agarwal and Said, 2003; O'Brien and Zini, 2005), including factors such as deficient chromatin packing (Manicardi *et al.*, 1995), abortive apoptosis (Shen *et al.*, 2002), environmental pollutants (Aitken, 2004) and increased oxidative stress (Aitken and Fisher, 1994). Sperm are particularly susceptible to damage by excessive levels of oxidative stress, due to their high content of unsaturated fatty acids and relative lack of cytosolic antioxidant protection (Aitken *et al.*, 2003a,b). The absence of DNA repair mechanisms further exacerbates this effect.

The importance of mtDNA quality in male fertility has also been increasingly recognized (Cummins *et al.*, 1994; St John *et al.*, 2005), with mtDNA deletions being associated with impaired sperm motility and infertility (Lestienne *et al.*, 1997; Kao *et al.*, 1998; Spiropoulos *et al.*, 2002). MtDNA is subject to much greater oxidative stress than nDNA due, in part, to its close proximity to respiratory chain complexes, which produce reactive oxygen species as a by-product of

oxidative phosphorylation (Van Houten *et al.*, 2006). The lack of histone protection (Shoffner and Wallace, 1994) also renders mtDNA more vulnerable to oxidative damage. Rapid replication, inefficient proof reading and limited repair mechanisms result in mutation rates that are 10–100 times higher than those found in nDNA (Kao *et al.*, 1998). Furthermore, damage to mtDNA in sperm has been shown to occur at much lower levels of oxidative stress than nDNA (Bennetts and Aitken, 2005) reinforcing its importance as a sensitive indicator of ‘sperm health’ (Lewis *et al.*, 2004).

Oxidative stress is also recognized to be an important factor in the pathogenesis of many of the chronic complications of diabetes (Giugliano *et al.*, 1996; Nishikawa *et al.*, 2000; Piconi *et al.*, 2003; Wiernsperger, 2003). Indeed, DNA damage in the diabetic vasculature is an important stimulus for the initiation of mechanisms resulting in endothelial dysfunction and ensuing vasculopathy. We hypothesize that the observed increase in sperm DNA damage is a further complication of diabetes in men whose developing sperm are exposed to supra-physiological levels of glucose and, therefore, oxidative insult.

In this study, control subjects were recruited from men attending for a semen analysis as part of a general infertility workup. These men were chosen due to the practical difficulty encountered in recruiting men of recent proven fertility. It could be argued that the current control group is not representative of the general population. However, given the association between infertility and both nDNA and mtDNA damage, one would reasonably expect these men, if anything, to be biased towards a higher level of nDNA fragmentation (Gandini *et al.*, 2000; Spano *et al.*, 2000; Zini *et al.*, 2001; Saleh *et al.*, 2002; Sergerie *et al.*, 2005) than their proven fertile counterparts. Therefore, any significant differences demonstrated between diabetic men and this control group would be of even greater significance if compared with a fertile population.

Conclusion

The effects of diabetes on human male reproductive function have, thus far, been largely neglected beyond concerns about impotence. Although this study shows that, other than semen volume, conventional semen parameters of diabetic men do not differ significantly from control subjects, their sperm do have increased levels of nDNA and mtDNA damage. From a clinical perspective this is important, particularly given the overwhelming evidence that sperm DNA damage impairs male fertility and reproductive health. Further studies characterizing the precise nature of this damage, the aetiological mechanisms behind it and evaluating its clinical significance are required

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