Insulin Dependant Diabetes Mellitus: Implications for Male Reproductive Function.

Agbaje, I. M., Rogers, D., McVicar, C., McClure, N., Mallidis, C., Atkinson, A. B., & Lewis, S. (2007). Insulin Dependant Diabetes Mellitus: Implications for Male Reproductive Function. Human reproduction, 22 (7)(7), 1871-1877. https://doi.org/10.1093/humrep/dem077

Published in:
Human reproduction

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Journal:	*Human Reproduction*
Manuscript ID:	HUMREP-06-1252.R1
Manuscript Type:	Original Articles
Date Submitted by the Author:	n/a
Complete List of Authors:	Agbaje, Ishola; Queens University Belfast, Reproductive Medicine Research Group, Centre for Clinical and Population Sciences Rogers, Deirdre; Queens University Belfast, Reproductive Medicine Research Group, Centre for Clinical and Population Sciences McVicar, Carmel; Queens University Belfast, Reproductive Medicine Research Group, Centre for Clinical and Population Sciences McClure, Neil; Queens University Belfast, Reproductive Medicine Research Group, Centre for Clinical and Population Sciences; Royal Maternity Hospital Belfast, Regional Fertility Centre Atkinson, A; Royal Victoria Hospital Belfast, Regional Centre for Endocrinology and Diabetes Mallidis, Con; Queens University Belfast, Reproductive Medicine Research Group, Centre for Clinical and Population Sciences Lewis, Sheena E M; School of Medicine, Obstetrics and Gynaecology, The Queens University of Belfast; Queens University Belfast, Reproductive Medicine Research Group, Centre for Clinical and Population Sciences
Keywords:	DIABETES MELLITUS, SPERM, DNA DAMAGE, MALE INFERTILITY
Specialty:	Andrology


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Insulin Dependant Diabetes Mellitus: Implications for Male Reproductive Function

Running Title: Diabetes impairs sperm DNA

*Agbaje I.M.[1], Rogers D.A.[1], McVicar C.M.[1], McClure N.[1,3], Atkinson A.B.[2], Mallidis C.[1], and Lewis S. E. M.[1]

[1]Reproductive Medicine Research Group, Centre for Clinical and Population Sciences, Queen's University of Belfast, Institute of Clinical Science, Grosvenor Road, Belfast, BT12 6BJ
Tel: +44 (0) 28 9063 5060
Fax: +44 (0) 28 9032 8247

[2]Regional Centre for Endocrinology and Diabetes, Royal Victoria Hospital, Grosvenor Road, Belfast, BT12 6BA.

[3]Regional Fertility Centre, Royal Maternity Hospital, Grosvenor Road, Belfast, BT12 6BA

*To whom correspondence should be addressed. E-mail: i.agbaje@qub.ac.uk

Key words: Diabetes Mellitus / human sperm / nuclear DNA / mitochondrial DNA / male infertility

BACKGROUND: Diabetes mellitus is increasing in men of reproductive age. Despite this, the prevalence of diabetes in men attending UK infertility clinics is unknown. Furthermore, studies examining its effects on sperm fertility potential have been limited to conventional semen analysis.

METHODS: The prevalence of diabetes mellitus in infertile men was determined by a postal questionnaire sent to all UK Human Fertilisation and Embryology Authority (HFEA) registered fertility clinics. Conventional semen analysis was performed for 27 diabetic and 29 non-diabetic subjects. Nuclear DNA fragmentation was assessed using the alkaline COMET assay and mitochondrial DNA deletions by long-polymerase chain reaction.

RESULTS: Fifty five percent of clinics responded to the questionnaire but only 2 (4%) were able to provide prevalence data for diabetes in infertile men. Other than a small, but significant, reduction in semen volume (2.6ml vs 3.3ml; $p<0.05$) conventional semen parameters did not differ significantly from control subjects. Diabetic subjects had significantly higher mean nuclear DNA fragmentation (53% vs 32%; $p<0.0001$) and median number of mitochondrial DNA deletions (4 vs 3; $p<0.05$) compared to control subjects.

CONCLUSIONS: Diabetes is associated with increased sperm nuclear and mitochondrial DNA damage which may impair the reproductive capability in these men.

Introduction

Diabetes mellitus (DM) represents one of the greatest threats to modern global health. Its incidence is rising rapidly. In the year 2000, 177 million people were affected by diabetes worldwide but by 2025 this figure is projected to rise to over 300 million (World Health Organization 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 *et al.* 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, making diabetes the commonest endocrine disorder in children (Silink 2002).

Type 2 diabetes, classically a late onset condition, is now being recognised much more frequently in younger age groups. In the United States alone, it is estimated to account for up to 76% of diabetes in the teenage years (Liese *et al.* 2006). As a consequence of these epidemiological changes for both type 1 & 2 diabetes many more men will be affected prior to and during their reproductive years.

Diabetes mellitus may affect male reproductive function at multiple levels as a consequence of its effects on the endocrine control of spermatogenesis, spermatogenesis itself or by impairing penile erection and ejaculation (Sexton *et al.* 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 *et al.* 1990a; 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 *et al.* 1997). Diabetes is, however, a well-recognised cause of male sexual dysfunction, which in itself may contribute to sub-fertility. Erectile dysfunction is more common in diabetic men (McCulloch *et al.* 1980; Feldman *et al.* 1994; Glenn *et al.* 2003) and occurs at an earlier age than in non-diabetic men (Klein *et al.* 1996). In addition, up to a third of diabetic men are estimated to suffer from ejaculatory disorders (Dunsmuir *et al.* 1996).

Studies of sperm quality in diabetes have been limited to light microscopic assessment of conventional semen parameters (semen volume, sperm count, motility and morphology). Classic semen analysis is now recognised 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.* 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. The potential impact of the increase in diabetes in young men and its effect on their reproductive health have 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 *et al.* 1997) and an increase in adverse reproductive outcomes such as miscarriage 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 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 *et al.* 2003; O'Brien *et al.* 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 DNA quality in sperm from diabetic and non-diabetic men.

The aim of this study was to compare male fertility potential by conventional and molecular techniques in diabetic and non-diabetic subjects.

Materials & Methods

Survey of Human Fertilisation and Embryology Authority (HFEA) Registered Fertility Units

A survey was performed (initially electronic and subsequently by postal questionnaire), in 87 fertility units holding a licence from the Human Fertilisation and Embryology Authority (HFEA). The questionnaire was designed to ascertain both the prevalence of DM (type 1&2) and associated semen abnormalities in infertility patients over a 1-year period.

Assessment of Semen Profiles and Sperm nDNA and mtDNA Quality

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. A venous blood sample was taken at the time of semen analysis for the measurement of Glycosylated Haemaglobin (HbA1c) in order 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 (ORECNI) 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 (World Health Organization 1999). Sperm morphology was assessed according to Tygerberg Strict Criteria (Kruger *et al.* 1988). Semen analysis was performed within 1 hour 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 minutes. 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.

Nuclear DNA 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 x $10^6 mL^{-1}$. 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 100μl 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 x 50 mm). Slides were left at ambient temperature (18°C) to allow the agarose to solidify. The coverslips were removed, and 10μl of diluted semen (6 x 10^6mL^{-1}) was mixed with 75μl 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 22.5mls of fresh lysis solution (2.5M NaCl, 100mM Ethylenediamene tetraacetic [EDTA] and 10mM Tris (pH10), with 1% Triton X-100 (Sigma) added just prior to use), for 1 hour at 4°C. Subsequently 10mM dithiothreitol [DTT] (Sigma) was added for a further 30 minutes at 4°C, followed by 4mM lithium diiodosalicylate [LIS] (Sigma) at ambient temperature for 90 minutes.

Unwinding of DNA

Slides were removed from the lysis solution and drained of any residual fluid. Fresh alkaline electrophoresis solution was prepared (300mM NaOH,

1mM EDTA; Sigma) and poured into a horizontal gel electrophoresis tank. The agarose coated slides were placed side by side in the tank, with the labelled end facing the cathode for 20 minutes, allowing the exposed DNA to unwind.

Separation of DNA Fragments by Electrophoresis

Electrophoresis was carried out for 10 minutes at 25V, with the current adjusted to 300mA, 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 neutralisation buffer (0.4M Tris; pH 7.5; Sigma), removing any residual alkali or detergents that may interfere with staining. Slides were stained with 50μl of 20μg/ml ethidium bromide (Sigma), covered with a glass coverslip and stored in a humidified container in darkness at 4°C, 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 100W mercury lamp and a barrier filter of 590nm. The proportion of fragmented DNA was determined using an image analysis system (Komet 3.1, Kinetic Imaging, Nottingham, United Kingdom) to analyse 50 sperm per slide (Hughes *et al.* 1997).

Assessment of Sperm Mitochondrial DNA Deletions by Long-Polymerase Chain Reaction

Sperm DNA Isolation

DNA was isolated from sperm samples using a Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN). Briefly, samples previously frozen in liquid nitrogen were allowed to defrost at room temperature and then centrifuged at 16000g for 1 minute to pellet cells. The supernatant was removed and 300μl of cell Lysis Solution (Gentra) added and pipette mixed. Following this, 12μl of 1M DTT (Sigma) and 1.5μl of 20mgmL^{-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, 1.5μl of RNAse A solution (Gentra) was added to the cell lysate and incubated for 1 hour at 37°C. Samples were again allowed to cool to room temperature prior to adding 100μl of Protein Precipitation Solution (Gentra). This was placed on ice for 5 minutes and then centrifuged at 16000g for 4 minutes to pellet the precipitated proteins. DNA was precipitated by pouring the supernatant containing the DNA into an Eppendorf tube containing 300μl of 100% isopropanol (Sigma) and inverting 50 times. Following centrifugation at 16000g for 1 minute and removal of supernatant, the tube was inverted on absorbent paper to drain for 15 minutes. The DNA pellet was subsequently washed with 300μl 70% Ethanol (Sigma) by inversion several times before centrifugation at 16000g for 1 minute. The supernatant was removed and the DNA pellet allowed to dry.

Following this, DNA was re-hydrated by adding 50μl of DNA Hydration Solution (Gentra) to the tube and incubating for 1 hour at 65°C.

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

Long-Polymerase Chain Reaction amplification

L-PCR amplification of mtDNA was performed in 50μl volumes using Bio-X-Act DNA polymerase (Bioline, London, UK) and a Hyabid touchdown thermal cycling system (Hyabid Ltd, Middlesex, UK). Reaction mixtures contained 1 x Optiform buffer (Bioline), 1.5mM $MgCl_2$, 0.25mM dNTPs, 500ng DNA template, 2U of Bio-X-act (Bioline) and 0.5μM 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 minutes, followed by 34 cycles of denaturation at 94°C for 10 seconds, annealing at 52°C for 30 seconds and extension at 68°C for 10 minutes. 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. Negative and positive controls were included in each set of reactions using primers for β-Actin. L-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 Tris-Acetate-EDTA buffer (10X TAE Buffer; Gibco-BRL, Life

Technologies, Paisley, United Kingdom), containing 1μgmL^{-1} ethidium bromide (Sigma). A voltage of 120V was applied for 60 minutes. Following electrophoresis, mtDNA deletions were visualised 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 L-PCR products. The deletion size was calculated by comparing its position on the gel to 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 SPSS 11 for Mac OS 10 (SPSS Inc., Chicago, Illinois, www.SPSS.com). Semen profiles and nuclear DNA fragmentation data from controls and diabetic men were compared using Student's t-test. Sperm concentrations and total sperm output were normalised using a square root transformation. To account for the non-Gaussian distribution of the mitochondrial DNA data, the non-parametric Mann Whitney-U test was used to compare median values for control and diabetic subjects.

Results

Survey of HFEA Registered Units

Questionnaires were returned from 48 of 87 (55%) centres offering fertility treatment. Only 2 (2%) were able to determine the prevalence of diabetes in male patients attending with infertility, which was reported as 0.2 and 1% respectively.

Comparison of Conventional Semen Profiles from Control and diabetic subjects

Semen samples from non-diabetic control and diabetic groups were compared (Table I). As expected, mean glycosyated haemoglobin was significantly higher in the diabetic group. There was no significant difference between groups in the mean age of subjects. Mean abstinence times did not differ between the control and diabetic groups. Mean semen volume in diabetic men was significantly less than for non-diabetic controls. However, no significant differences were observed in sperm count, total sperm output, percentage motility, or percentage normal morphology.

Nuclear DNA 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 to that from non-diabetic controls (n=23) (53%±3 *vs.* 32%±2;

p<0.0001) [Figure 1]. Our group has previously reported an Intra assay coefficient of variation less than 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 to controls (n=21) (4 [3-6] *vs.* 3 [1-4]; p<0.05) [Figure 2a]. 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 to 67% of controls (p<0.05). The median size of mtDNA deletions did not differ significantly between the two groups (7kb [6-7] *vs.* 7 [6-7]; p>0.05) [Figure 2b].

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 1 in 6 couples requiring specialist investigation or treatment in order to conceive (Hull *et al.* 1985; Schmidt *et al.* 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 sub-fertile men at approximately 1% (Greenberg *et al.* 1978; Sexton *et al.* 1997). Based on the background prevalence of DM and male infertility in this age group this figure was expected to be around 0.3% (Sexton *et al.* 1997). Despite this, our postal survey 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 to investigate 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 and colleagues found only semen volume and total sperm output to be significantly lower in diabetic men (Handelsman *et al.* 1985). In a larger study of 100 type-1 and 314 type 2 diabetics (Ali *et al.* 1993), the authors found an increase in sperm concentration and total sperm output but a concomitant reduction in motility and no difference in sperm morphology. Vignon et al. demonstrated higher sperm concentrations and abnormal morphology with no difference in motility (Vignon *et al.* 1991). Not surprisingly, many of these diabetic men with normal semen parameters had fathered children and the study concluded that DM, in itself, was not a cause of sub-fertility.

A study of 54 diabetic men (9 type-1 diabetics) found the principal abnormality to be a reduction in the forward motility of sperm (Dinulovic *et al.* 1990b). In contrast, one report of sperm motility (Niven *et al.* 1995), using

computer assisted image analysis, reported an increase in the linear movement of sperm from diabetic men. This group postulated that DM could even confer a minor advantage in terms of fertility, although the *in vivo* significance of this finding was not clear. In all 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 *et al.* 1997).

Conventional semen analysis remains core to the evaluation of male fertility in the clinical setting. However, whilst 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 sub-fertile (Bonde *et al.* 1998; Saleh *et al.* 2002). In addition, large intra-individual variations occur over time (Mallidis *et al.* 1991; World Health Organization 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 World Health Organisation (World Health Organization 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 nuclear and mitochondrial DNA 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 miscarriage 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 *et al.* 2005; Evenson *et al.* 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 *et al.* 1999a, 1999b). The oocyte has a limited ability to repair damaged sperm DNA (Matsuda *et al.* 1989; Genesca *et al.* 1992) and fragmentation beyond this threshold may result in increased rates of embryonic failure and pregnancy loss (Ahmadi *et al.* 1999b). In the context

of spontaneous conception, sperm DNA quality has been found to be poorer in couples with a history of spontaneous miscarriage (Carrell *et al.* 2003a; Carrell *et al.* 2003b).

Perhaps more worryingly, increased sperm DNA damage has been implicated in the future health of resulting offspring (Brinkworth 2000; Aitken *et al.* 2003a; 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 exists 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, amongst others, reinforce the value of these tests assessing the genomic integrity of sperm in the prediction of male fertility potential (Agarwal *et al.* 2005).

The aetiology of sperm DNA damage is multi-factorial (Agarwal *et al.* 2003; O'Brien *et al.* 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 *et al.* 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.* 2003b). The absence of DNA repair mechanisms further exacerbates this effect.

The importance of mtDNA quality in male fertility has also been increasingly recognised (Cummins *et al.* 1994; St John *et al.* 2005), with mtDNA deletions being associated with impaired sperm motility and fertility (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.* 2005). The lack of histone protection (Shoffner *et al.* 1994) also renders it 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 *et al.* 2005) reinforcing its importance as a sensitive indicator of 'sperm health' (Lewis *et al.* 2004).

Oxidative stress is also recognised 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 hypothesise 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 & mtDNA damage, one would reasonably expect these men, if anything, to be biased towards a higher level of nuclear DNA 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 to a fertile population.

Conclusion

The effects of diabetes on human male reproductive function have, thus far, been largely neglected beyond concerns about impotence. Whilst this study shows that 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 characterising the precise nature of this damage, the aetiological mechanisms behind it and evaluating its clinical significance are required.

Acknowledgements

The authors would like to thank Mrs Margaret Kennedy, Biomedical Scientist, Andrology Laboratory, Royal Jubilee Maternity Service, Belfast and the staff of the Regional Centre for Endocrinology and Diabetes, Royal Victoria Hospital, Belfast, for their help with this study.

Ishola Agbaje is a clinical research fellow in the Reproductive Medicine Research Group, Queens University Belfast funded by the Northern Ireland Research and Development Office (Grant no. EAT 2539). The authors are also members of the Recognised Research Group in Endocrinology and Diabetes of this office.

Legend

Figure 1

Values represent mean percentage fragmented DNA (SEM)

* p<0.0001; student's t-test

Figure 2

(a) mt DNA deletion number

(b) mean mtDNA deletion size

* p<0.05; Mann-Whitney-U

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Table I

	Group			
	Control (n=29)	**Diabetic** (n=27)	***p***	‡***WHO Normal***
†Age (years)	32.7 ± 0.7	34.0 ± 2.0	0.52	
†HbA1c (%)	5.3 ± 0.1	8.2 ± 0.2	<0.0001	
†Semen Volume (mL)	3.3 ± 0.2	2.6 ± 0.3	<0.05	2-4
††Sperm Concentration ($10^6 mL^{-1}$)	51 [28 – 100]	64 [30 – 151]	0.22	>20
††Total Sperm Output (10^6)	173 [89 – 338]	198 [99 – 450]	0.84	-
†Motility (%)	47.3 ± 2.8	46.0 ± 4.2	0.79	>50
†Normal Morphology (%)	11.7 ± 0.8	11.1 ± 0.6	0.56	>14

Comparison of Age, HbA1c & semen profiles from control and diabetic men

†Values expressed as mean ± SEM

†† Values expressed as median [inter-quartile range]

‡ World Health Organisation Normal Reference Values (WHO, 1999)

Fig 1. Comparison of nDNA fragmentation in sperm from control and diabetic men