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Advanced Glycation End Products AGEs) accumulate in the Reproductive Tract of Men with Diabetes.

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Abstract

Light microscopic studies comparing sperm parameters show little association between diabetes and male fertility. However, with the introduction of new analytical techniques, evidence is now emerging of previously undetectable affects of diabetes on sperm function. Specifically, a recent study has found significantly higher sperm nuclear DNA (nDNA) fragmentation in diabetic men. As advanced glycation end products (AGEs) are important instigators of oxidative stress and cell dysfunction in numerous diabetic complications, we hypothesized that these compounds could also be present in the male reproductive tract. The presence and localization of the most prominent AGE, carboxymethyl-lysine (CML), in the human testis, epididymis and sperm was determined by immunohistochemistry. Parallel ELISA and Western blot analyses were performed to ascertain the amount of CML in seminal plasma and sperm from 13 diabetic and 9 non-diabetic subjects. CML immunoreactivity was found through out the seminiferous epithelium, the nuclei of spermatogonia and spermatocytes, in the basal and principle cells cytoplasm and nuclei) of the caput epididymis and on most sperm tails, mid pieces and all cytoplasmic droplets. The acrosomal cap, especially the equatorial band, was prominently stained in diabetic samples only. The amount of CML was significantly higher p = 0.004) in sperm from non diabetic men. Considering the known detrimental actions of AGEs in other organs, the presence, location and quantity of CML, particularly the increased expression found in diabetic men, suggests that these compounds may play a hitherto unrecognized role in male infertility.

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Introduction

Diabetes mellitus continues to be a major healthcare issue, as both type 1 and type 2 forms of the condition are associated with a range of complications many due to the high incidence of macro and microvascular disease. As well as a general concern over the increasing frequency of diabetes in the Western world, there is particular alarm at the decreasing age of patients at diagnosis, especially in view of the long term health repercussions of the condition.

Concurrently, much has been written about falling fertility rates in industrialized countries. Numerous causes including social and environmental factors have been proposed for the phenomenon (Carlsen et al. 1992, Morgan. 2003, Skakkebaek et al. 2006). Much of this attention has been focused on impaired male reproductive function, in particular lowered sperm counts and decreases in sperm quality (Jensen et al. 2002, de Kretser. 1996).

Considering the prominence of diabetes and male sub fertility, and their potential ramifications for reproductive health at both the personal and public levels, it is surprising to note how little new information is available on any possible associations between diabetes and male infertility. Some previous studies have examined possible links between diabetes and various semen parameters (sperm concentration, motility, morphology etc). However, the overall findings are often contradictory and usually inconclusive (Ali et al. 1993, Handelsman et al. 1985, Niven et al. 1995, Vignon et al. 1991). It is not surprising therefore, that few infertility clinics even record the diabetic status of the male partner in couples seeking treatment for their childlessness.

A recent study reported a 1.6 fold increase in the percentage of fragmented sperm nuclear DNA nDNA) from men with type 1 diabetes compared to sperm from non- diabetic controls (Agbaje et al. 2007). Despite being associated with decreased embryo quality, lower implantation rates and a higher miscarriage rate (Henkel et al. 2004, Lewis & Aitken. 2005, Morris et al. 2002), the

assessment of sperm nDNA damage is not routinely included during the investigation of male fertility, as it is laborious, time consuming and clear boundaries of normality have not been established (Trisini et al. 2004).

The major cause of sperm nDNA damage is considered to be oxidative stress encountered during transit through and storage in the epididymis (Fouchecourt et al. 2000, Vernet et al. 2004). Sperm nDNA is particularly susceptible to attack by reactive oxygen species (ROS), due to their high unsaturated fatty acid content and the absence of DNA repair mechanisms (Aitken & Sawyer. 2003). Although numerous ROS types (e.g. hydrogen peroxide, hydroxyl radical), sources (e.g. leukocytes, immature or degenerating sperm) and laboratory procedures (e.g. centrifugation, cryopreservation) associated with ROS production, have been shown to cause sperm nDNA fragmentation (Aitken & Baker. 2006, Alvarez et al. 2002, Donnelly et al. 2001, Gil-Guzman et al. 2001) as yet the main factor responsible for the generation of nDNA damaging ROS in the male reproductive tract has not been identified.

Advanced glycation end products (AGEs) have been implicated as key pathogenic factors in the initiation and progression of many diabetic complications (Vlassara and Palace. 2002). These adducts form on the amino groups of proteins, on lipids and on DNA through a number of complex pathways including a non-enzymatic glycation by glucose and reaction with ascorbate, metabolic intermediates and reactive dicarbonyl intermediates (Singh et al. 2001). AGE adduct formation can modify the structure and function of proteins and may lead to complex crosslink formation. Metal catalysed oxidative reactions also give rise to a group of AGEs, classified as "glycoxidation" products such as N^{ε}-carboxymethyl-lysine (CML) and N^{ε}-carboxyethyl-lysine (CEL) (Wautier & Schmidt. 2004).

Accumulating during early embryonic development (Peppa et al. 2003), AGEs form during normal metabolic processes and are also introduced to the body, via a variety of exogenous routes including diet and smoking (Koschinsky et al. 1997, Vlassara & Palace. 2002). These adducts modify normal protein function and are capable of damaging DNA directly (Vlassara & Palace. 2002) or by generating ROS, either independently (Wautier & Schmidt. 2004) or via the auspices of a variety of receptors (Chekir et al. 2006, Schmidt et al. 2000). The binding of AGE ligands to the receptor for AGEs (RAGE) provokes a range of pathophysiological responses in many cell types which are linked to the downstream activation of various signaling pathways that in turn, lead to the production of ROS (Ramasamy et al. 2005).

AGEs have been found both to result from and to activate oxidative stress in various organs, either directly or, through the induction of ROS producing enzymes (Wautier & Guillausseau. 2001). Therefore we hypothesized that they may also be present in the male reproductive tract where they could possibly play a contributory role in the ROS initiated damage of sperm nDNA. The aims of this study therefore, were to determine whether one of the most prominent AGEs (CML) is present in the male reproductive tract, identify its location and to compare the levels of CML protein in the semen of men with type 1 diabetes and those of non- diabetic men.

Materials and Methods

Subjects

Written informed consent for participation was obtained and the project was approved by the local research ethics committee and Royal Group of Hospitals Trust Clinical Governance Committee.

Male type-1 diabetics attending the Regional Centre for Endocrinology and Diabetes, Royal Victoria Hospital, Belfast were invited to participate in this study (n = 13). Control samples were

obtained from men attending the QUB Andrology Laboratory for semen analysis as part of routine infertility investigations (n = 9).

Semen samples were collected after 2-5 days of sexual abstinence. All samples had conventional light microscopic analysis performed according to WHO recommendations WHO. 1999) for semen volume, sperm concentration and motility. Sperm morphology was assessed according to the Tygerberg Strict Criteria (Menkveld and Kruger. 1995). Semen analysis was performed within 1 hour of ejaculation after incubation at 37°C to allow for liquefaction to occur.

After analysis, the remainder of each sample was centrifuged at 300g for 10 minutes, the seminal plasma removed, frozen in liquid nitrogen and stored. The pellet was washed in an equal volume of phosphate-buffered saline (PBS) (Sigma Aldrich, UK) and centrifuged as before. After discarding the supernatant, the pellet was resuspended in PBS and apart from 10 μl used to prepare smears for immunocytochemistry, was frozen in liquid nitrogen and stored until needed for protein extraction. Sperm smears were prepared on 3-aminopropyltriethoxysilane (APEs) coated microscope slides, left to air dry, then fixed in absolute ethanol, air dried and stored until use.

Testicular biopsies were obtained from non- diabetic normogonadotrophic men (n=5) undergoing investigation for azoospermia at the Department of Urology and Pediatric Urology, Justus Liebig University, Giessen. Tissue was collected by open biopsy procedure, immediately immersed in Bouin's fixative, then washed, dehydrated through a series of graded ethanol, cleared in xylene and embedded in paraffin using standard techniques. Serial sections of 5 μm of each sample were cut and placed on APEs coated glass microscope slides.

Immunolocalization

Tissue sections were dewaxed, rehydrated using a sequence of xylene, graded ethanol and water before washing in PBS. The immunodetection procedure employed was a modification of the method previously described by Stitt et al. 2002). Briefly, antigen unmasking was performed by incubating the slides with a 50% trypsin: 50% versene solution Cambrex Bioscience, Berkshire, UK) for 2 minutes. After being washed in water for 20 minutes, the tissue was permeabilized in 0.1% Triton X-100 (Sigma Aldrich, UK) for 20 minutes, rinsed in PBS, and blocked with normal goat serum overnight at 4°C in a humidified chamber. The next day the slides were again washed in PBS, then incubated with 1:200 dilution of a previously validated (Reddy et al. 1995) rabbit anti-CML (gift of Professor S Thorpe, North Carolina) or rabbit Isotype control (Zymed Laboratories Inc, CA, USA) at similar concentration overnight at 4°C. An additional negative control, the omission of primary antibody, was also included. Finally the samples were washed in PBS, incubated with a donkey anti-goat Alexa 488 fluor antibody (1:200) (Invitrogen, Paisley, UK), washed again, incubated with propidium iodide for nuclear staining (1:200) for 20 mins at room temperature, washed, mounted and coverslipped. All slides were then examined and evaluated using a Bio-Rad Microradiance confocal scanning microscope (CSLM) fitted to an Olympus BX690 fluorescent microscope.

Other than the omission of the antigen unmasking step, the procedure used for the immunodetection of CML on sperm was identical to that detailed above.

Protein Extraction and Quantitation

Stored semen samples were thawed, aliquots of 20x 10^6 sperm taken and centrifuged at 16,000g for 15 minutes to fully separate sperm and seminal plasma. The seminal plasma was removed and reserved. A volume of extraction buffer (0.1% Triton X, 1% Tergitol, 0.1% SDS &

176 0.001% NaN_3) in PBS was added and the pellet sonicated on ice for 30sec using a hand held
177 mini pestle (Sigma Aldrich, UK). The resultant suspension was centrifuged at 10,000g for 10
178 minutes and the supernatant collected. Protein content of the sperm extracts and seminal
179 plasmas was determined using a Bichoninic acid kit (Pierce BCA™ Protein Assay Kit, Rockford,
180 IL) and microplate reader set at an absorbance of 562 nm.
181
182 ***ELISA***

183 CML-immunoreactivity was quantified using a modification of the standard competitive ELISA
184 protocol. Briefly, 200µl of 1µg/ml AGE-BSA (Cambridge Biosciences, Cambridge, UK) in 0.05M
185 carbonate buffer pH 9.6) was added in each well of a 96 well plate (Nunc™ Maxisorb,
186 Denmark), covered and incubated at 4°C overnight. Next day the plates were washed in PBS
187 wash buffer (2mM KH_2PO4, 3mM NaCl, 4.5µM 2,4-Hexadienoic acid potassium salt, 0.001%
188 Tween-20), blocked (3% skimmed milk in ddH_2O for 2 hours), washed, samples and standards
189 (duplicate wells of each) added, covered and incubated with 1:2000 dilution of 4G9, a previously
190 validated (Virella et al. 2005) monoclonal anti-CML antibody (2µg/ml) (gift of Professor S
191 Thorpe, North Carolina) with gentle agitation for 2 hours. Standards of AGE-BSA in protein
192 extraction buffer were serially diluted to give a range from 200µg to 0.39µg. Samples were
193 diluted in a 0.05% Tween 20, 0.2% BSA, 75nM PBS (pH 7.4) solution. A 1:5000 dilution of
194 peroxidase conjugated anti-mouse IgG was added, the plates covered and incubated for 1hour.
195 Following a final washing step, a 1:1 hydrogen peroxide-tetramethylbenzidine (TMB) solution
196 Sigma Aldrich, UK) was added and the plates returned to the dark with gentle agitation for 20
197 minutes. The color reaction was stopped by the addition of 2N H_2SO_4. All incubations were
198 conducted at room temperature. The optical density of each well was measured using a Tecan
199 Safire microplate reader (Tecan, UK) set to 450nm and corrected by subtracting the reading

obtained at 540nm. The concentration of CML was determined using the appropriate standard curve and standardized according to the total protein added to each well.

Western Blot Analysis

Extracted proteins were resuspended in 4X NuPAGE Buffer (Invitrogen, UK), heated to 100^{0}C for 5 mins then vortexed and centrifuged (13,000 rpm, 10 mins, RT). Triplicate aliquots (20 µg/ml per well) of each sample were loaded onto a native (4% stacking/12% resolving) gel and electrophoresed at 50V for 30 mins then 120 V for 90 minutes using the Mini Protean 3 system BioRad, UK). Upon completion, the separated proteins were transferred to a polyvinylidene PVDF) membrane (PALL Lifesciences, USA) using a Mini-Trans-Blot Cell (Biorad, UK). All subsequent steps were performed with gentle agitation and all dilutions and washes were performed using 0.01% PBS-Tween (PBST) (Santa Cruz Biotechnologies, USA). Following transfer, membranes were blocked (18 hours at 4^{o}C) with 5 % Blotto, then incubated with a CML polyclonal antibody (1:10,000) (gift of Professor S Thorpe, North Carolina) for 1hour prior to being washed (3 x 5min). The membranes were then incubated with goat anti rabbit- horse radish peroxidase (GAR-HRP) conjugated antibody (1:5000), (Sigma, Aldrich) for 1hour, washed then incubated (5 minutes at RT) with WestPico Chemiluminescent Substrate (Pierce, UK). Any peroxidase activity was visualized, documented and the intensity of each band quantitated, using the UVP AutoChemi System (MSC, Dublin, Eire). For verification of comparable loading, blots were stripped and reprobed using an identical procedure except for □-actin monoclonal antibody (1:3,000, Sigma Aldrich, UK) and goat anti mouse-horse radish peroxidase conjugated antibody GAM-HRP) (1:5000, Santa Cruz Biotechnologies, USA)

Statistical Analysis

Statistical analysis was performed using SPSS 11 for MAC OS 10 SPSS INC., Chicago, Illinois, www.spss.com). Semen profiles were compared using the Student's t-test. To account for the

non Gaussian distribution of total sperm count and ELISA data, a Mann Whitney U Test was used to compare median values for diabetic and non-diabetic subjects.

Results

Semen Analysis

No statistically significant difference was found in age diabetics 33.0 ± 3.8 years: non-diabetics 32.0 ± 1.3 years (mean ± standard error of mean), or most aspects of semen quality (Table 1) between the diabetic and control subjects. Non statistically significant differences in sperm concentration (decreased in non-diabetics) and motility (decreased in diabetics) were seen. A statistically significant increase (p=0.041) in total sperm count in non-diabetics was also noted. However all measures obtained were well above the normal limits, recommended by the WHO (WHO. 1999). A statistically significant difference was found in the HbA1c percentages diabetics (7.97 ± 0.41%, non-diabetics 5.37 ± 0.09%, p>0.0001).

Distribution of CML in the Testis and Epididymis

The immunocytochemical localization of CML in the testis (Figure 1a) showed a speckled staining pattern throughout the cytoplasm of all cell types present in the seminiferous epithelium. The intensity of the staining was particularly prominent around the basement membrane (spermatogonia) and midway to the tubule lumen (spermatocytes). The luminal space of the tubules was devoid of immunofluorescence other than that associated with early spermatogenic cells (both cytoplasm and nuclei) that had been either released prematurely or displaced as a consequence of the biopsy procedure. Apart from a slight background staining, no immunoreactivity was evident in the cytoplasm of the peritubular cells or the constituents of the interstitium. The nuclei of spermatogenic cells prior to the first meiotic division all presented a granular appearance suggesting the presence of CML within the nuclei. In particular, a

number of primary spermatocytes showed a heightened intensity of staining. The nuclei of round spermatids were clearly stained though not as intensely as less mature germ cells. Elongated spermatids, myoid peritubular cells and Leydig Cells did not present any immunoreactivity for CML.

Sections of the efferent ducts/caput region of the epididymis displayed various degrees of immunoreactivity, which was separated into distinct compartments (Figure 1b). In the regions of the epithelium with the more intense staining, the most prominent immunofluorescence was seen in the cytoplasm surrounding the basal cell nuclei, in the nuclei of the principal cells and a discrete band lining the lumen of the duct, suggestive of a greater quantity of CML in the ciliated apical domain of the principal cells. The smooth muscle layer encircling the tubule was diffusely stained and interspersed with non immunoreactive nuclei primarily of myoid cell origin but possibly also fibroblasts. Neither the basal lamina, nor the dense connective tissue beyond the smooth muscle ring, displayed any staining. The fluid contents of the blood vessels however showed an opaque uniform staining. The negative control sections showed no fluorescent staining (data not shown).

Localization of CML on Sperm

In samples from both diabetic and non-diabetic men, a speckled staining pattern extending the entire length of the tail and mid piece regions was detected in almost all sperm (Figure 2a). Though rare in samples from non- diabetics (Figure 2a - c), on the majority of sperm from diabetic men there was a particularly prominent immunofluorescence on the head corresponding to the acrosomal cap (Figure 2d - f). An equatorial band just below the acrosome was also very intensely stained and clearly delineated (Figure 2d - f) in most immunoreacted sperm regardless of their morphology (Figure 2f). Whenever present, cytoplasmic droplets, on

sperm from both non-diabetic (Figure 2c) and diabetic (Figure 2d - e) subjects, were intensely and uniformly stained. The negative control sections showed no immunoreactivity (Figure 2g).

ELISA

A significantly higher ($p = 0.004$) amount of CML protein was detected by ELISA in the sperm of non- diabetic men compared to that found in samples from diabetic men (Figure 3). The mean amount of CML in seminal plasma was greater than in sperm (35% in non- diabetics and 50% in diabetics) but there was no significant difference in the quantity between subject groups.

Western Blot Analysis

Consistent with the findings of the ELISA, samples from non-diabetic patients showed evidence of enhanced levels of CML-immunoreactivity when compared to diabetics. The banding pattern (Figure 4) showed a range of CML-modified proteins that were found in all sperm samples, however the majority were present in larger quantities in the non-diabetic than in the diabetic samples (e.g. 86, 64, 37, 23 kDa). A number of protein bands were seen only in non- diabetic samples (e.g. 54, 29 and 13 kDa), the most prominent being a strong and distinct band at approximately 11 kDa.

Discussion

Any skepticism surrounding the possible association between diabetes mellitus and impaired male reproductive function owes as much to paucity of number and scale of relevant studies, as it does to the dearth of definitive correlations. Based exclusively on the comparison of data obtained from routine semen analysis, the overall findings of previous studies are inconclusive. As a result, diabetes mellitus has been largely ignored as a relevant factor in male fertility assessment by the majority of fertility specialists.

Our demonstration of the lack of any difference in the routinely assessed parameters of sperm from diabetic and non- diabetic subjects is in accordance with previous studies (Bartak. 1979, Padron et al. 1984, Paz et al. 1977). However, beyond simple microscopic assessment, we have previously reported, a significant increase in the percentage of fragmented nDNA in samples from diabetic men Agbaje et al. 2007). This relatively new parameter is being given increasing prominence in the investigation of male infertility (Makhlouf & Niederberger. 2006) as the integrity of sperm nDNA has been shown to be associated with impaired embryonic development, increased incidence of spontaneous abortion and possibly, the onset of certain childhood diseases (Lewis & Aitken. 2005). No relationship has been established between microscopically assessed semen characteristics and nDNA fragmentation (Saleh et al. 2002, Trisini et al. 2004). In fact our findings of increased levels of nDNA fragmentation but normal semen analysis parameters are consistent with similar results for infertile men (Saleh et al. 2002), adolescents with varicocele (Bertolla et al. 2006), men over the age of 45 (Moskovtsev et al. 2006) and cigarette smokers (Sepaniak et al. 2006).

Although the precise cause of sperm nDNA damage remains elusive, the current prevailing view is that the fragmentation occurs as a result of oxidative stress due to excessive ROS generation caused by aberrant mitochondrial function, cytoplasmic remnants, inadequate control of testicular heating and/or leukocyte contamination, amongst other factors (Lewis & Aitken. 2005). However, other than increased levels of leukocytes in the semen of cigarette smokers (Close et al. 1990), and, possibly elevated testicular temperature in men with varicocele (Turner. 2001) there is little evidence that any of these factors are increased in diabetes or any of the other aforementioned conditions.

Formation of AGEs is an established pathogenic mechanism in diabetic complications (Peppa & Vlassara. 2005, Vlassara & Palace. 2002). As part of the normal aging process (Chavakis et al.

2004), AGEs accrue at a heightened rate during hyperglycemia (Peppa & Vlassara. 2005) and may form very rapidly depending on the presence of dicarbonyls, the availability of metal ions and changes in oxidative radicals (Han et al. 2005, Sajithlal et al. 1998). The immunocytochemical localization of CML-immunoreactivity in the cytoplasm of all cell types within the seminiferous epithelium suggests that they are susceptible to adduct formation. This implies that, differentiating spermatozoa may be influenced by some form of AGE instigated/mediated cellular events) throughout their development although this requires further mechanistic investigation. The presence of CML in the nuclei of pre mitotic cells also opens the possibility that the later encountered sperm nDNA damage may not be the result of oxidative attack alone but could also be due to the destabilization or fragmentation of the DNA by direct AGE modification of nuclear proteins (Gugliucci & Bendayan. 1995) which may undermine DNA integrity and lead to strand breaks (Vlassara & Palace. 2002) during nDNA compaction in the caput epididymis. This phenomenon could provide a possible explanation for the increased number of ultrastructurally “immature” sperm with uncondensed DNA encountered in diabetic samples (Baccetti et al. 2002), and also seen in our own unpublished study.

Our study has found that the basal and principal cells of the caput epididymis, accumulate CML, and since this is an organ known for its absorptive and phagocytotic capabilities, it is tempting to speculate that this indicates a possible mechanism for the removal of senescent AGE-modified proteins. Indeed, a similar phenomenon has been demonstrated in the tubular epithelium of the kidney (Alderson et al. 2004, Bohlender et al. 2005). This would represent a defensive response which is necessary in the caput, as it is here that sperm undergo major modifications, especially the final compaction of their nDNA. However, the staining pattern reported could, just as likely, indicate the local generation of new AGEs involving pre existing proteins (Bohlender et al. 2005).This in turn would facilitate the heightened expression of and binding to RAGE. An event which amplifies the damaging effects of AGEs (Ramasamy et al. 2005) and may contribute to or

mediate the propagation and/or maintenance of a potentially hostile environment in the epididymis. For example, there is a large body of evidence showing that under a variety of settings, AGE-RAGE binding can trigger the rapid generation of ROS (Ramasamy et al. 2005), via the activation of NADPH oxidase amongst others processes (Wautier & Guillausseau. 2001), which results in the ongoing generation of oxidative stress and hence sustained production of ROS and AGEs (Wendt et al. 2003). This is of particular interest when considering that sperm spend a substantial amount of time in the epididymis where they are particularly vulnerable to oxidative attack and that the epididymis is thought to be the primary site of ROS induced sperm nDNA damage (O'Connell et al. 2002, Suganuma et al. 2005).

Considering the ubiquitous distribution of AGEs seen in the seminiferous epithelium, it is not surprising that the proteins comprising the sperm tail should show the presence of CML. The speckled appearance of the staining merely reflects the number of tail proteins containing glycated lysine groups. However, it is the CML localization on the acrosomal cap and the equatorial region of the sperm head, primarily seen in diabetic samples, that is particularly noteworthy as it is this area, especially the acrosomal cap itself, that undergoes numerous modifications during epididymal maturation, and which constitutes a direct access point to the underlying nDNA. It may also be the site of potentially the greatest external AGE induced damage. Beyond their ability to instigate an oxidative cascade, AGEs may also facilitate attack from ROS produced by other mechanisms (e.g. leukocytes, cytoplasmic droplets and degenerating sperm) by weakening the plasma membrane encapsulating and protecting the genome.

The increased CML-immunoreactivity found in sperm and seminal plasma of non- diabetic compared to diabetic samples, may at first seem counter intuitive to the proposed actions of AGEs. However, considering that the main site of nDNA fragmentation is in the epididymis, the

findings in ejaculated sperm may reflect the results of either the heightened levels of deglycation mechanisms or possibly enhanced AGEs elimination processes triggered by the chronic diabetic state. Actions which are belatedly successful in removing AGEs, predominantly from the tail, but are unable to prevent or repair the already completed nDNA damage. The situation is further confused by the sperm's immersion in seminal plasma, a fluid consisting of a myriad of proteins and glycoproteins in the presence of fructose, a more potent AGE forming hexose than glucose (Schalkwijk et al. 2004). A truer indicator of AGE quantity and damage potential would be evaluation of AGE adducts derived from a range of dicarbonyl intermediates. Similarly, the evaluation of epididymal, ideally caput, sperm and the localization of AGE binding proteins would be a useful adjunct to the current study and is undergoing investigation in our laboratory.

Finally, the roles played by AGEs in diverse processes such as tissue damage, cell death, inflammatory response, oxidative stress and DNA fragmentation in numerous organs and cells, suggest that the presence of CML in the testis, epididymis and on spermatozoa may be a portent that these complex compounds could contribute to a multitude of similar but as yet unexplained conditions which ultimately result in male sub fertility. Consequently they form a new path worthy of significant further investigation in both diabetic and non diabetic men alike.

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602 **Figure Legends**

603 **Figure 1**:

604 Immunohistochemical localization.

605 The testis: **a)** a speckled fluorescent staining green) through out the cytoplasm of all cells of the
606 seminiferous epithelium. Most cells close to the basal membrane of the tubules and midway to
607 the lumen displayed a more intense immunoreactivity around the nuclei (thick arrows). The
608 nuclei of some primary spermatocytes were particularly immunoreactive (dotted arrows) while
609 most elongated spermatids contained no staining (thin arrow).
610 The epididymis: **b)** Immunoreactivity in the efferent ducts/caput region was separated into
611 distinct compartments of the epithelium. In the areas with the most immunofluorescence, the
612 cytoplasm surrounding the basal cell nuclei, (dashed arrow), the nuclei of the principal cells
613 solid arrow) and a discrete band lining the lumen of the duct (thin arrow) were more intensely
614 stained. The smooth muscle layer encircling the tubule was diffusely stained (asterisk) whilst the
615 fluid contents of the blood vessels dotted arrow) displayed an opaque uniform immunoreactivity.
616

617 **Figure 2**:

618 Immunocytochemical localization of CML on sperm. On the head region of the spermatozoa
619 appearance extending from the mid section to the edge of the head and the distinct equatorial
620 band just below the acrosome.
621 **a)** Low magnification of a representative sample from a non diabetic subject showing the
622 characteristic (green) immunofluuorescence on the length and mid piece regions of almost all
623 sperm and occasionally on the head (arrow). **b)** Higher magnification of individual sperm from
624 non diabetic showing the speckled staining and **c)** the immunoreactivity of any cytoplasmic
625 droplets (asterisk). Higher magnification of sperm from diabetic subjects showing
626 immunofluorescence on the acrosomal cap and the prominent staining of the equatorial band

(arrow) and cytoplasmic droplets (asterisk) (**d & e)** regardless of head morphology (**f).** The negative control sections showed no fluorescent staining (**g)**.

Figure 3:

ELISA quantitation of CML in sperm filled bars) and seminal plasma open bars) from non diabetic and diabetic patients. *Significantly different ($p = 0.004$). Error bars signify SEM

Figure 4

Western blot analysis of sperm proteins. Upper panel shows characteristic banding pattern with non-diabetic sample (N) displaying larger quantities of numerous proteins (dotted arrows) than the diabetic sample (D). A number of protein bands were seen only in the non- diabetic samples (solid arrows). s corresponds to the weight marker and the lower panel the β actin loading control

Table 1

Sperm characteristics of participating subjects as determined by routine microscopic semen analysis

	Volume (ml) †	Sperm Concentration (10^6)††	Total Sperm Number (10^6)††	Motility (%)†	Morphology (%)†
Diabetics	3.3 ± 0.6	129 (62– 273)	450 (270-553)*	49.8 ± 0.8	12.0 ± 0.6
Non Diabetic	3.1 ± 0.3	77 (36-108)	218 1(01-339)	53.1 ± 4.7	13.8 ± 1.3

† Values expressed as means ± standard error of the means

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

* p =0.041

Figure 1

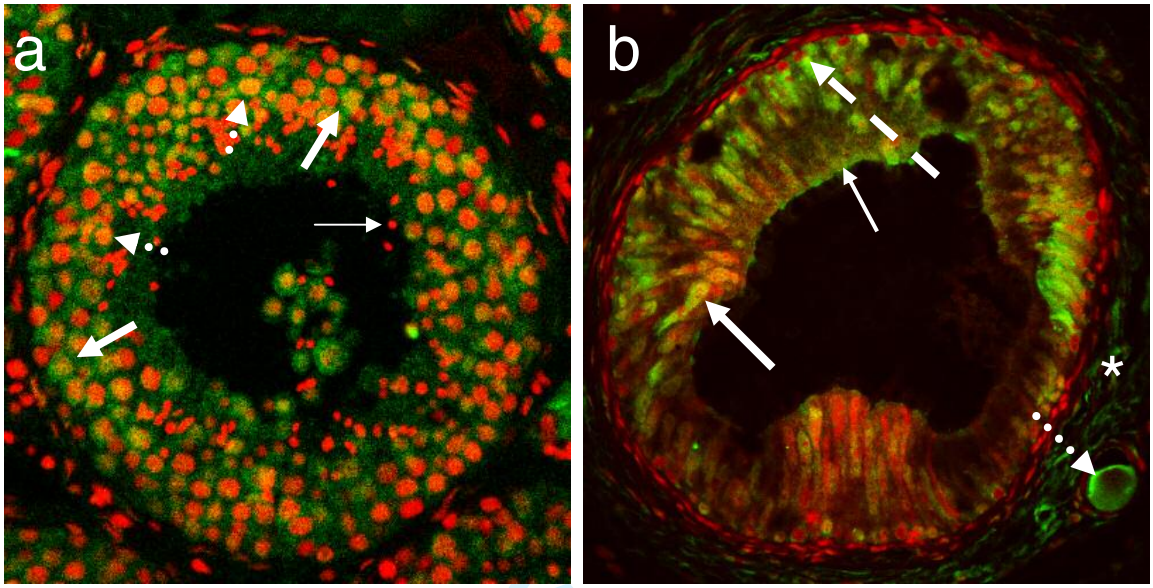

Figure 2

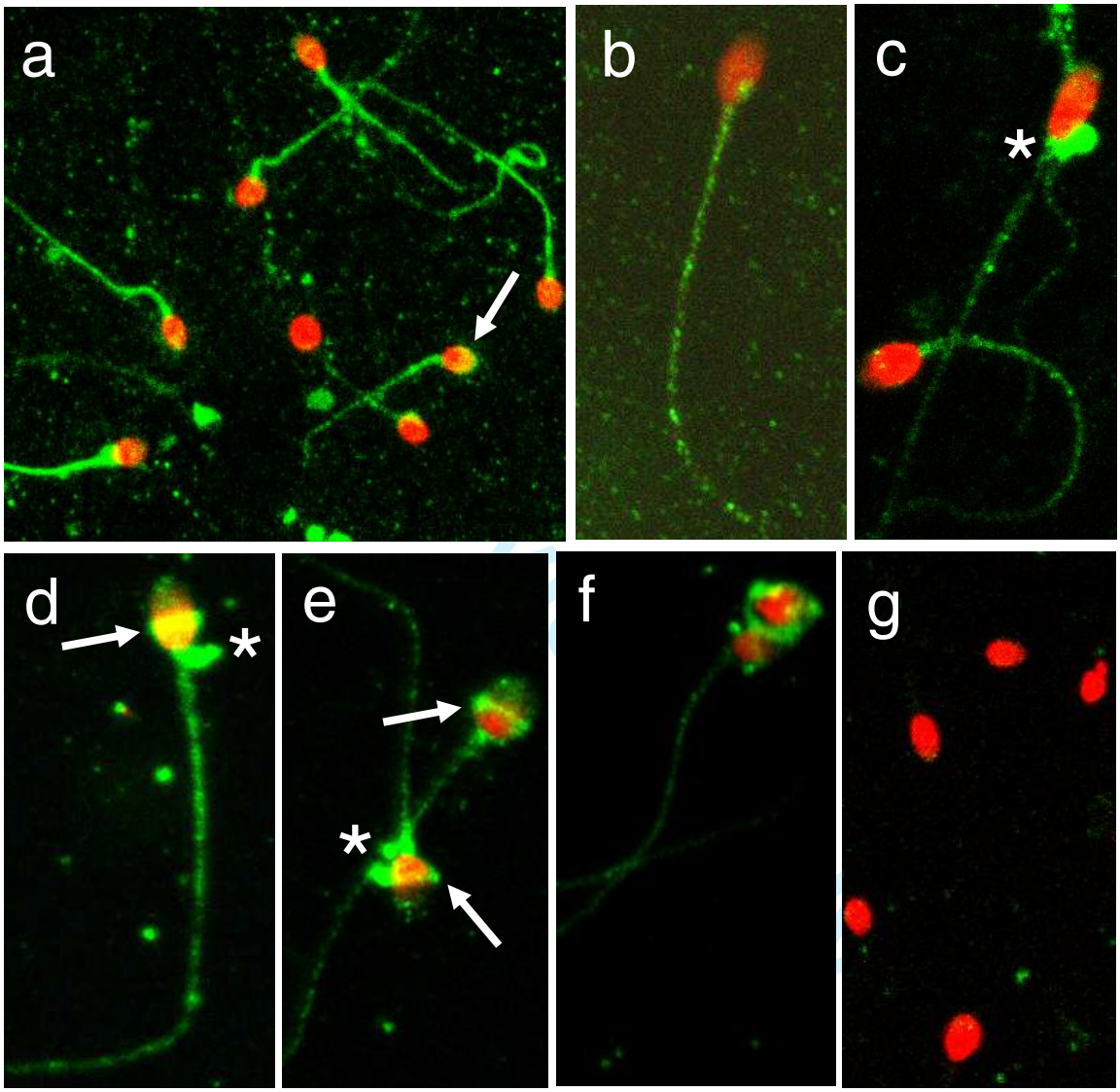

Figure 3

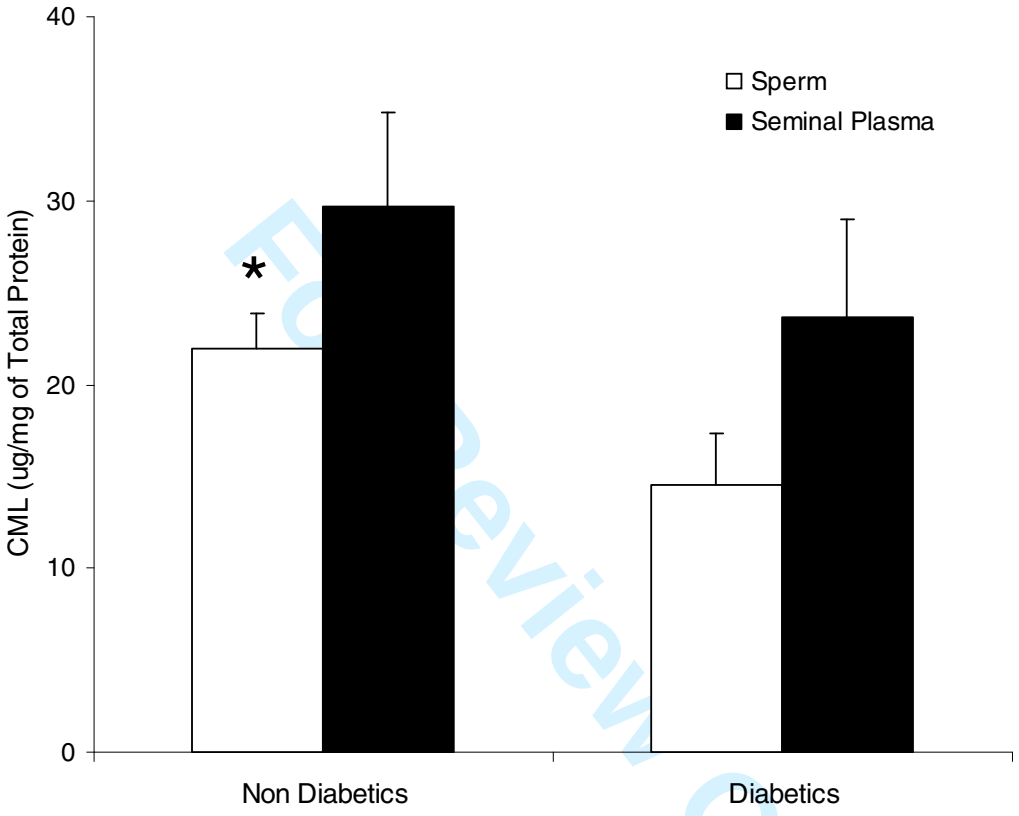

Figure 4