J Assist Reprod Genet (2013) 30:843–848
DOI 10.1007/s10815-013-0015-0

GAMETE BIOLOGY

High prevalence of isolated sperm DNA damage in infertile men with advanced paternal age

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Received: 30 March 2013 / Accepted: 17 May 2013 / Published online: 1 June 2013
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Abstract
Background Sperm DNA damage is associated with male infertility, lower pregnancy rates and pregnancy loss.
Objective The primary aim of our study was to evaluate the prevalence of sperm DNA damage in younger and older men with normozoospermia.
Design, Setting and Participants We obtained semen from 277 consecutive non-azoospermic men presenting for sperm DNA testing.
Outcome Measurements and Statistical Analysis The main outcome measures included sperm % DNA fragmentation index (%DFI, using sperm chromatin structure assay), sperm concentration, motility and morphology, and, paternal age.
Results and Limitations Sperm % DFI was positively correlated with paternal age (r=0.20, P<0.001) and inversely correlated % progressive motility (r=−0.16, P=0.01). Sperm %DFI was significantly higher in older (≥40 years) compared to younger (<40 years) normozoospermic men (17±13 vs. 12±8, respectively P=0.008), whereas, sperm concentration, progressive motility and morphology were not significantly different in these two groups. Moreover, the prevalence of high levels of sperm DNA damage (>30 % DFI) was significantly higher in older compared to younger normozoospermic men (17 % vs. 3 %, respectively, P<0.001).
Conclusion The data indicate that a conventional semen analysis can often fail to detect a defect in spermatogenesis (high %DFI) in older men and suggest that infertile couples with advanced paternal age, including those with normal semen parameters, should consider sperm DNA testing as part of the couple evaluation.

Keywords Sperm · DNA fragmentation · Age · Normozoospermia · Infertility

Capsule In a retrospective study of 277 consecutive non-azoospermic men presenting for sperm DNA testing, we have found that the prevalence of high levels of sperm DNA damage was significantly higher in older (≥40 years) compared to younger (<40 years) normozoospermic men.

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Introduction

There is now good evidence to show that sperm DNA and chromatin defects are associated with male infertility and reduced natural conception rates [13, 17, 32]. Several studies have also demonstrated that sperm DNA damage is associated with advanced paternal age and poor semen parameters [11, 16, 26, 27, 34]. Increased levels of sperm DNA damage have been linked with chromosomal abnormalities, developmental loss and birth defects in mouse model systems [18, 24]. Various mechanisms have been proposed to explain the increase in sperm DNA damage with age, ranging from oxidative stress to inefficient apoptosis processes [31]. However,

there is a paucity of data regarding the prevalence of high levels of DNA damage in normozoospermic men and its relationship with paternal age.

A systematic review of the literature demonstrates that sperm DNA damage is associated with lower pregnancy rates after assisted conception techniques, namely, intrauterine insemination and in-vitro fertilization (IVF) and to a lesser extent with intracytoplasmic sperm injection (ICSI) [8, 9, 36]. Nonetheless, sperm DNA damage is associated with an increased risk of pregnancy loss after IVF and after ICSI, and because ICSI bypasses the natural defense barriers and allows for fertilization with DNA damaged sperm, there is growing concern regarding the health of the resulting offspring [14, 41].

The purpose of our study was to further evaluate the relationship(s) between sperm DNA damage, conventional semen parameters and paternal age. We specifically compared the prevalence of isolated sperm DNA damage in younger (<40) and older (≥40) men as this has not been previously reported.

Methods

Materials

Acridine orange (AO) was purchased from PolySciences (Warrington, PA). Unless otherwise stated, all other chemicals were obtained from Sigma Chemical Co (St. Louis, MO) and were reagent grade or higher.

Study subjects and semen handling

Semen samples were obtained from consecutive non-azoospermic men presenting for sperm DNA testing (n=277) at the Andrology Research laboratory of the Royal Victoria Hospital at McGill University. The two main indications for sperm DNA testing in these men were (1) evaluation of unexplained couple infertility and (2) prediction of IUI or IVF-ICSI outcomes. These indications were based on prior publications suggesting a potential clinical role for sperm DNA testing in the context of unexplained infertility and for prediction of IUI or IVF-ICSI outcomes [13, 32, 36, 41]. The institutional ethics review board at McGill University approved this study.

Samples were produced by masturbation after 3 to 5 days of sexual abstinence and allowed to liquefy at room temperature. After liquefaction of semen, standard sperm parameters (concentration, %progressive motility, %strict morphology) were obtained by manual technique as per recent WHO guidelines [10]. Infertile men were sub-classified according to the nature of the sperm parameter defect(s) in their semen samples as follows: normozoospermia (≥15 million sperm/ml and, ≥32 % with progressive motility and ≥4 % normal forms) and oligoasthenozoospermia (<15 million sperm/ml and/or <32 % with progressive motility and/or <4 % normal forms). Information on the age of the men at the time of the semen analysis was obtained.

Following liquefaction, a 25 to 100 μL aliquot of semen (containing approximately 2 million spermatozoa) was collected from the original sample and frozen at −70 °C for later evaluation of sperm % DNA fragmentation index (% DFI). All of the semen samples had motile sperm and none had significant numbers of round cells or leukocytospermia as per WHO guidelines (< 1 million round cells per ml).

Sperm DNA fragmentation index (DFI)

Sperm DNA damage was assessed by the sperm chromatin structure assay (SCSA) and the results were expressed as sperm % DFI (an index of DNA damage), as previously described [13, 39]. Stored semen samples were thawed on ice and treated for 30 s with 400 μL of a solution of 0.1 % Triton X-100, 0.15 M NaCl, and 0.08 N HCl, pH 1.2. After 30 s, 1.2 mL of staining buffer (6 μg/mL AO, 37 mM citric acid, 126 mM Na_2HPO_4, 1 mM disodium EDTA, 0.15 M NaCl, pH 6.0) was admixed to the test tube.

The sample was placed into the FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA) with the sample flowing to establish excellent sheath/sample flow, and then at exactly 3 min after AO staining measurements were taken. A minimum of 5,000 cells from two aliquots of each sample were acquired and analyzed by FACS scan interfaced with a data handler (CELLQUEST 3.1, Becton Dickinson) on a Power Macintosh 7600/132 computer (Cupertino, CA). WinList (Verity Softwarehouse Inc., Topsham, ME) was used to analyze the data, and, generate the cytogram (red vs. green fluorescence) and histogram (total cells vs. DFI) plots, as well as, % DFI readings. A mean of the two sperm % DFI values was reported. The variability of the replicate % DFI measures was less than 5 %.

We have shown that testing fresh and frozen-thawed samples gives comparable results (<5 % variability) and that the inter-assay variability of sperm % DFI is low (<5 %) by repeat assessments of reference semen samples [37, 38]. Over 300 aliquots of the same semen sample (“reference sample”) have been stored at –70 °C for ongoing assessment of inter-assay variability. We have previously validated our assay by assessing sperm DNA fragmentation in parallel with sperm % DFI and have shown a strong association (r=0.71) between these two measures of DNA damage [37].

Statistical analysis

We used the Shapiro-Wilks test to evaluate the distribution of the data. Comparisons were analyzed with the use of Student t test or Mann-Whitney U test when appropriate. Differences

between the subgroup of patients were estimated by ANOVA. Variables (e.g. age, sperm motility) were correlated with the dependent variable (% DFI) by univariate analysis (spearman rank order). All hypothesis testing was two-sided with a probability value of 0.05 deemed as significant. Statistical analysis was performed using Sigma Stat software (SPSS Inc, Chicago, USA). The main outcome measures included sperm % DNA fragmentation index (% DFI, using sperm chromatin structure assay), sperm concentration, motility and morphology, and paternal age.

Results

The mean (±SD) age, sperm concentration, % progressive motility, % normal forms (strict) and % DFI in the group of 277 men was 36.9±5.4 years, 68±63 million per ml, 45±22 %, 6±3 % and 14±12 %, respectively. Sperm %DFI was positively correlated with paternal age (r=0.20, P<0.001) and inversely correlated with %progressive motility (r=−0.16, P<0.001). There were no significant relationships between sperm % DFI and sperm concentration or % normal forms.

In the group of men with normozoospermia (≥15 million sperm/ml and, ≥32 % with progressive motility and ≥4 % normal forms), sperm % DFI was significantly higher in older (≥40 years) than in younger men (<40 years) (17±13 vs. 12±8, respectively P=0.008), whereas, sperm concentration, % progressive motility and % normal forms were not significantly different in these 2 groups (Table 1). The prevalence of high levels of sperm DNA damage (>30 % DFI) in normozoospermic men was significantly higher in the older compared to younger men (17 % vs. 3 %, respectively, P<0.001).

In the men with oligoasthenoteratozoospermia (<15 million sperm/ml and/or <32 % with progressive motility and/or <4 % normal forms), sperm % DFI was also significantly higher in older (≥40 years) than in younger men (<40 years) (20±18 vs. 12±10, respectively P=0.003). In this group, % progressive motility was significantly lower in older (≥40 years) than in younger men (<40 years) (24±14 vs. 30±16, respectively P=0.042) but sperm concentration and % normal forms were not significantly different in these 2 groups (Table 1).

Discussion

Our study demonstrates that sperm % DNA fragmentation index (%DFI) is positively correlated with age. The findings are in keeping with previous studies showing that advancing paternal age is linked to sperm chromatin defects (4, 5). Moskovtsev et al. [26] reported that DNA fragmentation was significantly higher in men more than 45 years old, with % DFI doubling in those men 45 years and older compared to those less than 30 years old. Their cohort consisted of men presenting for fertility investigation and included both fertile and infertile men. Spano and colleagues [32] reported that sperm DNA damage almost doubled from 25 to 55 years of age, in a study involving 215 first pregnancy planners with unknown fertility capability [32]. In support of these findings, Singh et al. [31] reported that the percentage of sperm with highly damaged DNA was significantly higher in men aged 36–57 years than in those aged 20–35 years. They also noted an age-related decrease in sperm apoptosis and proposed that the higher sperm DNA damage with increasing age could be the result of a less efficient sperm cell selection processes with age (11). In contrast, Nijs et al. [29] studied a cohort of couples undergoing IVF and reported that advanced paternal age is not associated with sperm DNA damage [29]. An important weakness of most studies relating to sperm DNA damage

Table 1 Standard sperm parameters and sperm % DFI in the 2 semen parameter subgroups and the 2 age subgroups (<40 years and ≥40 years)

Subgroup	Younger men (<40 years)	Older men (≥40 years)	P-Value
Normozoospermia			
Number of patients	107	41	
Sperm concentration (×106/mL)	95±68	99±58	P=0.50
Progressive sperm motility (%)	61±14	58±17	P=0.09
Sperm morphology (strict)	8±2	7±4	P=0.25
Sperm % DFI	12±8	17±13	P=0.008
Oligoasthenoteratozoospermia			
Number of patients	97	32	
Sperm concentration (×106/mL)	35±37	33±37	P=0.84
Progressive sperm motility (%)	30±16	24±14	P=0.042
Sperm morphology (strict)	4±3	4±2	P=0.63
Sperm % DFI	12±10	20±18	P=0.003

Values are means ± SD; DFI = DNA fragmentation index

and paternal age is that the patient populations are highly selected (i.e. infertile men). As such, it is unclear whether the younger and older cohorts have comparable severity of infertility with similar conventional semen parameters allowing for fair comparison of the two groups.

Advanced paternal age has been associated with reduced fertility and poor semen parameters, particularly, reduced semen volume, sperm motility and sperm morphology [12, 22]. Therefore, based on the published studies, it is unclear whether the relationship between paternal age and sperm DNA damage is independent of the relationship between paternal age and conventional semen parameters or the severity of infertility. As such, we chose to evaluate the relationship between age and sperm DNA damage in a subgroup of infertile men with normal semen parameters (to minimize the potential influence of conventional semen parameters on DNA damage). It is important to note that in our study, a large proportion of the men (53 %) had normozoospermia because many of these men presented with unexplained infertility or for IUI treatment. We found that the sperm % DFI was significantly higher in normozoospermic men with advanced age (≥40) compared to younger men (<40). We have also shown that the prevalence of high levels of sperm DNA damage (>30 % DFI) in normozoospermic men was significantly higher in the older compared to younger men (17 % vs. 3 %, respectively). These findings suggest that the relationship between paternal age and sperm DNA damage does not parallel the relationship between paternal age and conventional semen parameters and, moreover, that a conventional semen analysis alone may fail to uncover a defect in spermatogenesis in older men. We also suspect that the higher levels of sperm DNA damage in older men accounts, in part, for the higher prevalence of secondary infertility in older men, as we have previously reported in our infertile population [40].

Older men may produce more sperm with DNA damage as a consequence of an age-associated increase in reproductive tract oxidative stress and/or altered testicular germ cell apoptosis [3, 4, 6, 30]. Oxidative stress within the testis and reproductive tract can damage sperm DNA, as well as, the sperm mitochondrial and nuclear membranes [1]. Germ cell apoptosis during spermatogenesis is a normal event, however, this process may be less effective in older men resulting in the release of more DNA-damaged sperm [6, 30]. Indeed, the testes of older male mice have lower apoptotic frequencies than young adults [5]. While apoptosis has been identified in the testes of elderly men [6], there have been no comparisons on the rates of germ cell apoptosis among men of different ages.

The high levels of sperm DNA damage in older normozoospermic men may be associated with reduced male reproductive potential and, possibly, may also be an indicator of genetic (point) mutations [21]. Although the ASRM Practice Committee [25] does not recommend routine use of sperm DNA tests, several studies have demonstrated that poor sperm chromatin and DNA integrity is associated with a very low potential for natural fertility and a prolonged time to pregnancy [13, 17, 32]. Sperm DNA damage has been shown to adversely affect intrauterine insemination and to a lesser degree IVF pregnancy rates, but not IVF/ICSI pregnancy rates [9, 15, 35, 36]. Sperm DNA damage has also been associated with poor embryo development and quality [42]. Moreover, a systematic review and meta-analysis of IVF and ICSI studies shows that sperm DNA damage is associated with a significant increase in the rate of pregnancy loss after IVF and ICSI [36]. The increased rate of failed embryos and pregnancy loss associated with male factor infertility and sperm DNA damage in IVF and ICSI may be due to sperm chromosomal aneuploidy but this has not been demonstrated conclusively [7, 28]. It has been suggested that the process of sperm selection during ICSI in humans may lessen the potential undesirable effects of sperm DNA damage on fertility outcomes [15]. Nonetheless, because ICSI bypasses the natural defense barriers, the high levels of DNA damage in these men remains concerning (at least theoretically) as some men will become fathers. To date, it is unclear whether the level of sperm DNA fragmentation is lower in morphologically normal sperm selected from a sample with high levels of fragmentation, as the reports on this subject provide conflicting results [2, 19]. Therefore, selecting morphologically normal sperm from a sample with a high level of DNA fragmentation does not guarantee that the level of DNA fragmentation will be lower in the sperm used for ICSI.

The influence of sperm DNA damage on the health of the child is unknown because of the paucity of clinical (human) studies on this subject. In experimental studies on mice, sperm DNA damage has been associated with chromosomal abnormalities, developmental loss, reduced longevity and birth defects [14, 18, 24]. In humans, advanced paternal age is associated with rare autosomal dominant diseases such as achondroplasia and Apert's syndrome, both due to point mutations, but whether these mutations are related to the global integrity of the sperm DNA, as measured by tests such as the sperm chromatin structure assay, remains to be verified [21, 22].

In accordance with our earlier observations [38], we found that the percentage of sperm with DNA fragmentation (%DFI) was inversely correlated with progressive sperm motility, which lends support to the idea that sperm motility and chromatin integrity are related. Indeed, sperm chromatin integrity and motility share a common origin. The formation of a mature sperm nucleus, which is characterized by the replacement of nuclear histones with protamines, and the development of the sperm flagellum both originate during spermiogenesis. In agreement with our findings, several investigators

have reported that sperm % DFI is associated with sperm motility [11, 16, 20, 27, 33]. In contrast, others have reported either an absence of any meaningful correlation between SCSA parameters and World Health Organization (WHO) parameters [13] or have observed only a weak correlation between these parameters [23]. These differences may be due to the heterogeneous characteristics of the sample population as well as different methods of analysis. In our study, the correlation between sperm motility and % DFI was significant, although the correlation coefficient was low.

Conclusion

Our study demonstrates that sperm DNA damage is positively correlated with age. Moreover, our data indicate that the prevalence of isolated sperm DNA damage (>30 % DFI and normozoospermia) in infertile men is significantly higher in older compared to younger men (17 % vs. 4 %, respectively). Taken together, the data suggest that a conventional sperm analysis alone may fail to detect a defect in spermatogenesis in older men. Evaluation of sperm DNA damage may help to uncover a defect in spermatogenesis in older men and define the most appropriate method of assisted conception for these couples.

Funding No external funding was either sought or obtained for this study.

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