Which isolated sperm abnormality is most related to sperm DNA damage in men presenting for infertility evaluation

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Abstract

Background Sperm DNA damage is common in infertile men and is associated with poor semen parameters but the impact of an isolated sperm abnormality on sperm DNA damage has not been studied.

Objective To evaluate sperm DNA damage in a large cohort of infertile men with isolated sperm defects.

Design, setting and participants Retrospective study of 1084 consecutive, non-azoospermic infertile men with an isolated sperm defect: isolated oligozoospermia (iOligo), isolated asthenozoospermia (iAsthenozoospermia) or isolated teratozoospermia (iTerato).

Outcome measurements and statistical analysis We examined and compared clinical parameters, conventional semen parameters and %sperm DNA fragmentation (%SDF, assessed by flow cytometry-based Terminal deoxynucleotidyl

transferase-mediated dUTP Nick End-Labeling assay) in the three groups of men.

Results and limitations The mean ($\pm$ SD) %SDF was significantly higher in the iAsthenozoospermia compared to the iOligo and iTerato groups (25.0 ± 14.0 vs. 19.2 ± 11.6 and 20.7 ± 12.1 %, respectively, $P < 0.0001$). Similarly, the proportion of men with high %SDF (>30 %) was significantly higher in the iAsthenozoospermia compared to the iOligo and iTerato groups (31 % vs. 18 % and 19 %, respectively, $P < 0.0001$). In the group of 713 men with iAsthenozoospermia, %SDF was positively correlated with paternal age ($r = 0.20$, $P < 0.0001$) and inversely correlated with %progressive motility ($r = -0.18$, $P < 0.0001$). In the subset of 218 men with iTerato, %SDF was also positively correlated with paternal age ($r = 0.15$, $P = 0.018$) and inversely correlated with %progressive motility ($r = -0.26$, $P = 0.0001$).

Conclusions In this large cohort of infertile men with isolated sperm abnormalities, we have found that the sperm DNA fragmentation level is highest in the men with sperm motility defects and that 31 % of these men have high levels of sperm DNA fragmentation. The data indicate that poor motility is the sperm parameter abnormality most closely related to sperm DNA damage.

Capsule In a large retrospective study, we have found that men with isolated asthenozoospermia have significantly higher sperm DNA damage than men with isolated oligozoospermia or isolated teratozoospermia. The data indicate that sperm DNA damage is most closely related to sperm motility defects.

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Introduction

The etiology of male factor infertility remains poorly understood, with close to 50 % of all cases deemed idiopathic or unexplained [1]. Recently, studies have examined the factors that may induce sperm DNA damage in the hope that these observations will shed some light on the etiology and pathophysiology of male factor infertility. Human sperm DNA

damage may be due to primary or intrinsic defects in spermatogenesis (e.g. genetic or developmental abnormalities) or due to secondary or extrinsic factors causing testicular or post-testicular injury (e.g. gonadotoxins, hyperthermia, oxidants, endocrine abnormalities) [2–10]. Investigators have suggested that protamine deficiency (with aberrant chromatin remodeling), reactive oxygen species (ROS) and abortive apoptosis may cause sperm DNA damage [11–18]. De Iuliis et al, have proposed a two-step model to explain the origin of sperm DNA damage. In their model, De Iuliis et al, propose that oxidative stress (2nd step) acts on poorly protaminated spermatids (i.e. with incomplete replacement of histones by protamines and poor chromatin compaction) that are formed as a result of defective spermiogenesis (1st step) [19].

We now know that infertile men have higher levels of sperm DNA damage than fertile men [20–22]. Moreover, the extent of sperm DNA damage in infertile men increases as the number of sperm parameter abnormalities increases [20, 21]. However, it is still unclear which of the conventional sperm parameter abnormalities is most related to sperm DNA damage. Evaluating infertile men with isolated sperm defects may allow us to determine which specific sperm abnormality is most closely linked to sperm DNA damage and help further our understanding of the etiology of sperm DNA damage in infertile men.

The purpose of our study was to evaluate sperm DNA damage in a large cohort of infertile men with isolated sperm defects. We also sought to evaluate the relationship between sperm DNA damage, and, the clinical and semen parameters in these men.

Materials and methods

Materials

Hematoxyline solution and Schorr solution were purchased from RAL Diagnostics, Martillac, France. In Situ Cell Death Detection Kit, Fluorescein was purchased from Roche Diagnostics Corporation, Mannheim, Germany. Triton X-100 was from Supelco Inc, Bellefonte, PA, USA. Sodium citrate was purchased from Merck, KGaA, Darmstadt, Germany. Formaldehyde solution (37 %), DNase (5 mg/mL) and the other chemicals were from Sigma Aldrich, Steinheim, Germany.

Study population

This is a retrospective study of 4945 consecutive, non-azoospermic and non-severely oligozoospermic men presenting for semen analysis and sperm DNA testing at the Laboratoire d'Eylau-Unilabs (Paris, France) between January and October 2013. All of the men were being evaluated for couple infertility (defined as failure to achieve a natural

pregnancy after at least 1 year of unprotected intercourse) but we did not ascertain whether the couples had pure male factor infertility, pure female factor infertility or combined male-female factor infertility. Azoospermic and severely oligozoospermic men (<1 million sperm per ml) were not included because there was insufficient material to perform the flow cytometry-based sperm DNA fragmentation assay. We excluded 600 men that were on medication, smoked cigarettes, provided a semen sample with more than 7 days abstinence and/or had a recent febrile illness (in the 3 months preceding the semen analysis) from the original cohort of 4945 men. We recorded the age and BMI (body mass index) of these men. The work was approved by the local ethics review board. All participants were informed of the study and signed a consent form as per the ethics board guidelines.

Semen analysis

The participants were asked to provide semen samples by masturbation after 2–7 days of sexual abstinence. After liquefaction of semen, the semen analysis was performed according to the World Health Organization (WHO) guidelines and included assessment of semen volume, sperm concentration, % progressive motility. The % normal morphology was assessed according to the modified David classification after Harris-Schorr staining [23–25]. The David classification of morphological anomalies of human spermatozoa was developed in the 1970s and modified in the early 1990s to account for all of the defects known to interfere with normal sperm functions. The vast majority of public and private laboratories in France have adopted the David classification. It allows classification of seven anomalies of the head (tapered, thin, microcephalous, macrocephalous, multiple, abnormal or absent acrosome, abnormal postacrosome), three anomalies of the midpiece (cytoplasmic droplet, thin, bent) and five anomalies of the principal piece (absent, short, irregular, coiled and multiple).

We identified 3 sub-groups of patients with isolated sperm defects and these groups were categorized based on the new WHO reference values: isolated oligozoospermia (iOligo: <15 million sperm per ml, ≥ 32 % progressive motility and ≥ 4 % normal forms), isolated asthenozoospermia (iAsthenozo: <32 % progressive motility, ≥ 15 million sperm/ml and ≥ 4 % normal forms) and isolated teratozoospermia (iTerato: <4 % normal forms, ≥ 15 million sperm/ml and ≥ 32 % progressive motility) [23]. The morphology thresholds of 4 % was taken from new WHO semen analysis manual and was deemed appropriate given that the new WHO semen analysis morphology reference values were derived in large part from studies using the David morphology classification [23, 26, 27]. A 700 μ L aliquot of raw semen was collected from the original sample and stored at 4 °C for the subsequent evaluation of sperm DNA fragmentation.

Sperm DNA fragmentation

Sperm DNA damage was assessed by flow cytometry-based TUNEL assay (Terminal deoxynucleotidyl transferase-mediated dUTP Nick End-Labeling) and reported as %sperm DNA fragmentation or %SDF—reflecting the percentage of cells with DNA damage, as described previously [28]. Briefly, washed spermatozoa were fixed with formaldehyde and permeabilized with 0.1 % triton X-100 in 100 μ L of 0.1 % sodium citrate for 3 min at 4 °C. After washing, the labeling reaction (supplied with the In Situ Cell Death Detection Kit, Fluorescein) was performed with TdT enzyme for 45 min at 37 °C in the dark. Samples were washed and analyzed by flow cytometry with at least 5000 events recorded within the region characteristic of spermatozoa (FC 500, Beckman Coulter, Villepinte, France). A negative control (without TdT) was run together with each sample, and a positive control was run at the beginning of each series of samples (by treating sperm with DNase diluted in PBS). Sperm DNA fragmentation was determined as the percentage of sperm having fluorescence intensities above a threshold established in the negative control histogram. We have shown that repeat testing using our flow cytometry TUNEL assay gives comparable results with a low (<10 %) intra-assay variability (data not shown).

Data analysis

Results are expressed as mean $\pm$ SD. Inter-group differences in sperm parameters (e.g. between iAsthen, iOligo and iTerato subgroups) were assessed by ANOVA. The relationships between parameters were examined using linear regression analysis with Pearson's correlation coefficient. All hypothesis testing was two-sided with a probability value of 0.05 deemed as significant. The cutoff value for high %SDF (>30 %) was based on a prior publication [28]. Statistical analysis was performed using SPSS version 20 (SPSS Inc, Chicago, USA).

Results

We identified 1084 consecutive infertile men with isolated sperm defects: 713 men with iAsthen, 153 with iOligo and 218 with iTerato. Therefore, 25 % (1084/4345) of the total cohort of evaluable infertile men presented with an isolated sperm defect.

We observed that the mean %SDF was significantly higher in subgroup of men with iAsthen compared to the subgroups with iOligo and iTerato, respectively (25.1 $\pm$ 14.0 vs. 19.2 $\pm$ 11.6 and 20.7 $\pm$ 12.1 %, $P<0.00001$, see Table 1). Moreover, the proportion of men with high %SDF (>30 %) was significantly higher in subgroup of men with iAsthen compared to

Table 1 Mean ($\pm$ SD) clinical parameters, conventional semen parameters and %sperm DNA fragmentation (%SDF), and, the proportion of men with high sperm DNA fragmentation (%hSDF) in the subgroups of infertile men with isolated oligozoospermia (iOligo), asthenozoospermia (iAsthen) and teratozoospermia (iTerato)

Subgroup	iAsthen	iOligo	iTerato	<i>P</i> -value
<i>n</i>	713	153	218	
Age	38 $\pm$ 7 ^a	37 $\pm$ 6 ^a	37 $\pm$ 6 ^a	NS*
BMI (body mass index)	25 $\pm$ 3 ^a	25 $\pm$ 4 ^a	24 $\pm$ 3 ^a	NS*
Volume (ml)	3.1 $\pm$ 1.3 ^a	3.9 $\pm$ 1.9 ^a	3.2 $\pm$ 1.6 ^a	NS*
Concentration (million/ml)	46 $\pm$ 39 ^a	10 $\pm$ 4 ^b	52 $\pm$ 31 ^a	<0.00001*
Progressive motility (%)	23 $\pm$ 8 ^a	38 $\pm$ 5 ^b	42 $\pm$ 9 ^c	<0.00001*
Morphology (% normal)	11 $\pm$ 6 ^a	11 $\pm$ 6 ^a	2 $\pm$ 1 ^b	<0.00001*
%SDF	25.0 $\pm$ 14.0 ^a	19.2 $\pm$ 11.6 ^b	20.7 $\pm$ 12.1 ^b	<0.00001*
%hSDF	31% ^a	18% ^b	19% ^b	<0.00001**

^{a,b,c} Different letters indicate significant difference between subgroups

*Kruskal-Wallis one-way analysis of variance on ranks

**Chi-square analysis

NS not significant ($P\geq 0.05$)

the subgroups with iOligo and iTerato, respectively (31 % vs. 18 % and 19 %, $P<0.00001$, see Table 1).

In the group of 713 men with iAsthen, %SDF was positively correlated with paternal age ($r=0.20$, $P<0.0001$) and inversely correlated with %progressive motility ($r=-0.18$, $P<0.0001$). There were no significant correlations between sperm DNA fragmentation and sperm concentration ($r=-0.002$, $P>0.05$), sperm morphology ($r=-0.13$, $P>0.05$) or BMI ($r=0.02$, $P>0.05$) in the 713 men with iAsthen.

In the subset of 218 men with iTerato, %SDF was also positively correlated with paternal age ($r=0.15$, $P=0.018$) and inversely correlated with %progressive motility ($r=-0.26$, $P=0.0001$). There were no significant correlations between sperm DNA fragmentation and sperm concentration ($r=-0.13$, $P>0.05$), sperm morphology ($r=-0.12$, $P>0.05$) or BMI ($r=0.005$, $P>0.05$) in the 218 men with iTerato.

There were no significant correlations between sperm DNA fragmentation and %progressive motility ($r=-0.08$, $P>0.05$), sperm concentration ($r=0.14$, $P>0.05$), sperm morphology ($r=-0.10$, $P>0.05$), age ($r=0.04$, $P>0.05$) or BMI ($r=-0.04$, $P>0.05$) in the 153 men with iOligo.

Discussion

In this large study of infertile men, we have found that men with an isolated sperm motility defect have a significantly higher mean level of sperm DNA fragmentation than men with an isolated sperm concentration or morphology

abnormality. We have also shown that the proportion of men with high sperm DNA fragmentation is significantly higher in the subgroup of infertile men with isolated asthenozoospermia compared to the subgroups with isolated oligozoospermia or isolated teratozoospermia. Moreover, in the subgroups of men with isolated asthenozoospermia and those with isolated teratozoospermia, sperm DNA fragmentation is inversely related to sperm progressive motility but is not related to sperm concentration or morphology. Taken together, these data are in keeping with earlier observations demonstrating a significant relationship between sperm DNA damage and sperm motility [20, 21, 29–35].

The underlying nature of the relationship between sperm DNA damage and sperm motility has not been fully characterized. A possible explanation for this relationship is that sperm DNA integrity and sperm motility share a common origin during spermatogenesis. Indeed, the formation of a mature, compacted sperm nucleus (characterized by the replacement of nuclear histones with transition proteins and then protamines) and the development of the sperm flagellum, both originate during spermiogenesis. Moreover, experimental studies have demonstrated that targeted disruption of nuclear chromatin compaction (using protamine or transition protein insufficiency models) is associated with the development of an abnormal flagellum and defective motility [16, 36]. Another potential explanation for the observed association between DNA integrity and motility lies in the inherent susceptibility of human spermatozoa to oxidative stress. Investigators have shown that abnormal semen samples generate high levels of reactive oxygen species and that these same spermatozoa are susceptible to membrane lipid peroxidation and subsequent loss of motility [37–39]. The translocation of lipid peroxides to the nucleus can then result in damage to the DNA [40–43]. These findings are in line with the 2-step theory proposed by De Iuliis et al, where oxidative stress (2nd step) acts on poorly protaminated cells generated by defective spermiogenesis (1st step) and this leads to sperm DNA damage [44].

Our data demonstrate that advancing age is positively correlated to sperm DNA damage in the subgroups of infertile men with isolated asthenozoospermia and isolated teratozoospermia. The findings are in keeping with several studies showing that paternal age is linked to sperm DNA damage [45, 46]. Moskovtsev et al. (2006) reported that DNA fragmentation was significantly higher in men more than 45 years old compared to those less than 30 years of age [47]. Spano and colleagues reported that sperm DNA damage almost doubled from 25 to 55 years of age, in a study involving 215 first pregnancy planners [48]. Similarly, Singh et al (2003) noted that the percentage of sperm with damaged DNA was significantly higher in men over 35 years of age than in those 35 years and under [49]. They also found an age-related decline in sperm apoptosis and suggest that the higher sperm DNA damage with increasing age could be the result of

poorer intra-testicular sperm selection with ageing, whereby many abnormal testicular sperm fail to be eliminated by apoptosis [30]. In contrast, Nijs et al (2011) studied couples undergoing IVF and reported that advanced paternal age is not associated with sperm DNA damage [50]. Nonetheless, it is important to note that a weakness of the current study, as well as that of most published studies on the correlation between sperm DNA damage and paternal age, is that they include infertile men with abnormal semen parameters. Therefore, it is hard to ascertain that the relationship between sperm DNA damage and paternal age is independent of the relationship between sperm DNA damage and semen parameters.

Studies have reported that sperm DNA damage is associated with reduced male reproductive potential and may be a marker of genetic mutations arising during spermatogenesis [18, 51]. The ASRM Practice Committee [52] does not recommend the routine application of sperm DNA tests, but studies have shown that sperm DNA damage is predictive of a low potential for natural fertility and a prolonged time to pregnancy [22, 48, 53]. Furthermore, sperm DNA damage may adversely impact intra-uterine insemination outcomes and to a lesser degree IVF pregnancy rates, but not IVF/ICSI pregnancy rates [3, 10, 22, 48, 52–55]. Sperm DNA damage is also associated with a significantly increased risk of pregnancy loss after IVF and ICSI [56, 57]. To date, little is known regarding the effect of sperm DNA fragmentation on post-natal health because of the lack of clinical (human) studies on this subject [58]. In animal studies, sperm DNA damage is associated with chromosomal abnormalities, developmental loss, reduced longevity and birth defects [59]. In humans, paternal age is related to sperm chromatin damage and increased risk of de novo gene mutations in sperm but whether these mutations in sperm are related to the global integrity of the sperm DNA, as measured by tests such as the TUNEL assay, remains to be verified [18, 51, 60].

The current study clearly shows that infertile men with an isolated sperm motility defect will often (31 % of the time) demonstrate high levels of sperm DNA damage. In this study, we did not observe a significant relationship between sperm DNA damage and abnormal morphology. To date, the published data on the relationship between sperm DNA integrity and normal morphology are conflicting. Although some studies have shown that abnormal sperm morphology and DNA damage are related [20, 61] other studies have not identified a significant relationship between these parameters [34, 62]. Our data are convincing in view of the fact that the study involves a large cohort of infertile couples with very well defined sperm defects. This type of selection allows us to better characterize the relationship between sperm DNA damage and conventional sperm parameters. Moreover, unlike most other studies on the subject, we have excluded men that produced semen samples under circumstances that can introduce artefact or uncontrolled alterations in sperm parameters

and DNA integrity (e.g. prolonged abstinence, recent febrile illness, cigarette smoking, medications). However, a weakness of our study is that the impact of an isolated sperm motility defect (and associated sperm DNA damage) on fertility potential is unclear because we have not studied a cohort of fertile men and do not have pregnancy outcome data. Another potential weakness of our study is that sperm morphology was assessed using the David classification rather than strict (Tygerberg) criteria. However, we believe that the application of the 4 % thresholds (the 5th percentile from the new WHO reference range) is appropriate given that the new WHO semen analysis morphology reference values were derived in large part from studies using the David morphology classification [23, 25–27].

Conclusion

In this very large cohort of infertile men with isolated sperm abnormalities, we have found that the sperm DNA fragmentation level is highest in the men with sperm motility defects and that 31 % of these men have high levels of sperm DNA fragmentation. The data indicate that poor motility is the sperm parameter abnormality most closely related to sperm DNA fragmentation.

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