Article

The effects of male age on sperm DNA damage in an infertile population

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Abstract

The objective was to investigate the influence of age on sperm DNA damage. Semen samples were collected from 508 men in an unselected group of couples attending infertility investigation and treatment. DNA fragmentation in spermatozoa was measured by TdT (terminal deoxynucleotidyl transferase)-mediated dUTP nick-end labelling (TUNEL) assay; at least 200 spermatozoa in randomly selected areas of microscope slides were evaluated using a fluorescent microscope and the percentage of TUNEL positive spermatozoa was determined. The number of cells with red fluorescence (TUNEL positive) was expressed as a percentage of the total sample [DNA fragmentation index (DFI)]. Age was treated as a continuous variable for regression and correlation analysis. The following male age groups were used: Group I: ≤35 years, Group II: 36–39 years, and Group III: ≥40 years. DFI was significantly lower in Group I than in Group II ($P = 0.034$) or III ($P = 0.022$). There was no difference in DFI between Groups II and III. In addition, regression analysis demonstrated a significant increase in sperm DFI with age ($P = 0.02$). TUNEL assay clearly demonstrates an increase in sperm DNA damage with age.

Keywords: *age, DNA damage, male infertility, spermatozoa, TUNEL*

Introduction

Advanced paternal age has been implicated in the increased frequency of spontaneous abortions (de La Rochebrochard and Thonneau, 2002; Nybo Andersen et al., 2004; Slama et al., 2005; Kleinhaus et al., 2006), autosomal dominant disorders, aneuploidies, and other diseases (Zhang et al., 1999; Malaspina et al., 2001; Fisch et al., 2003; Glaser et al., 2003; Sloter et al., 2004; Lambert et al., 2006; Reichenberg et al., 2006; Wyrobek et al., 2006; Schmid et al., 2007). A plausible explanation for these findings is that older men may have more spermatozoa with damaged DNA. New approaches to evaluate chromosomal and genetic human sperm defects give more direct information for identifying paternal risk factors. Chromatin damage has been associated with male infertility and with problems with conception and sustained pregnancy (Carrell et al., 2003; Virro et al., 2004; Nasr-Esfahani et al., 2005; Borini et al., 2006; Erenpreiss et al., 2006; Tesarik et al., 2006; Zini and Libman 2006; Bungum et al., 2007; Ménézo et al., 2007). Also there is growing evidence associating sperm DNA damage with mutation development risks and offspring defects (Wyrobek et al., 2006; Schmid et al., 2007).

Sperm DNA damage can be revealed by the detection of multiple DNA strand breaks, similar to those resulting from programmed cell death. The most commonly used methods for revealing DNA fragmentation are based on detecting low-molecular-weight DNA fragments (Comet assay) or on visualizing endogenous nicks in the DNA molecule (sperm chromatin structure assay: SCSA) and TdT (terminal deoxynucleotidyl transferase)-mediated dUTP nick-end labelling (TUNEL) assay (Nasr-Esfahani *et al*., 2005; Tesarik *et al*., 2006; Zini and

Libman 2006; Ménézo *et al*., 2007). Understanding the effects of male age on sperm DNA damage is especially relevant for men attending reproductive clinics because of the increasing reliance on modern technologies, especially in marginally fertile older men. Some studies have demonstrated an increase in sperm DNA damage with age in healthy men (Spano *et al*., 1998; Singh *et al*., 2003; Wyrobek *et al*., 2006; Schmid *et al*., 2007), but there is less information on infertile ones (Sun *et al*., 1997; Morris *et al*., 2002; Moskovtsev *et al*., 2007).

Based on the above evidence, this study aimed to investigate the influence of age on sperm DNA damage in a group of men from an infertility population, using TUNEL assay.

Materials and methods

Study participants

Semen samples (one per subject) were obtained from 508 men from an unselected group of couples undergoing infertility investigation and treatment at the Centre for Human Reproduction Prof. Franco Jr.

Sample collection

Semen samples were collected in sterile containers by masturbation after a period of 2 to 5 days sexual abstinence. A portion of each semen sample was immediately taken and processed for DNA damage assessment. The liquefied fresh semen sample was centrifuged at 200 *g* for 10 min at room temperature to separate spermatozoa from seminal plasma. The sperm pellet was resuspended in 1 ml phosphate-buffered saline (PBS) and centrifuged at 200 *g* for 10 min. The resultant pellet was then sent for sperm DNA fragmentation analysis. The remainder of the semen sample was analysed for standard semen quality parameters according to World Health Organization (1999) protocols.

Determination of DNA damage

DNA fragmentation in spermatozoa was measured using the TUNEL assay, which was performed using a Cell Death Detection Kit with tetramethylrhodamine-labelled dUTP (Roche, Monza, Italy). TUNEL identifies single- and double - stranded DNA breaks by labelling the free 3'-OH termini with modified nucleotides in an enzymatic reaction with TdT. TdT polymerises free 3-OH DNA ends in a template-independent manner, incorporating labelled nucleotides. The remaining sperm pellets were smeared on glass slides, air-dried, fixed with 4% paraformaldehyde in PBS at 4°C for 25 min, pH7.4, and permeabilized with 0.1% Triton X-100 (VETEC Química Fina Ltd, Duque de Caxias, Brazil) in 0.1% sodium citrate at 4°C for 2 min. After washing with PBS, the smears were then processed for TUNEL assay. The TdT-labelled nucleotide mix was added to each slide and incubated in the dark in a humidified atmosphere for 2 h at 37°C. After stopping the enzyme reaction, slides were rinsed twice in PBS and then counterstained with Vectashield® Mounting Medium with 4,6-diamidino-2-phenylindole (DAPI; 1.5 µg/ml) (Vector Laboratories, Burlingame, CA, USA). For quantitative evaluation, at least 200 spermatozoa in randomly selected areas on microscope slides were evaluated using a fluorescent microscope and the percentage of TUNEL-positive spermatozoa determined. The number of cells per field stained with DAPI (blue) was counted first; the number of cells with red fluorescence (TUNEL positive) was expressed as a percentage of the total sample (DNA fragmentation index: DFI). Controls were included in every experiment: for negative control, TdT was omitted in the nucleotide mix. Positive controls were generated by pre-incubating the fixed and permeabilized sperm cells using DNaseI 1 mg/ml (New England Biolabs, Inc, Ipswich, MA, USA) for 30 min at 37°C. TUNEL labelling of positive controls varied between 89–98% of cells. The same technician, blinded to subject identity, performed all examinations.

Statistical analysis

Data were analysed using InStat version 3.0 (GraphPad Software, San Diego, CA, USA) on a Macintosh computer (Apple Computer Inc., Cupertino, CA, USA). The Kruskal–Wallis Test, Mann–Whitney *U* test, and chi-squared test were used, as appropriate. Correlations were performed using the Spearman rank correlation test. Age was treated as a continuous variable for regression and correlation analysis. For comparisons, subjects were divided into three groups by age: Group I: ≤ 35years; Group II: 36–39 years; and Group III: ≥40 years. The level of significance was set at $P < 0.05$.

Results

Table 1 summarizes the general characteristics of the study population. An equal distribution ($P > 0.05$) of the main characteristics was observed for all three groups (**Table 2**). The only exception was history of previous pregnancy: a significantly higher number of older men (≥ 40years, Group III) had fathered at least one child (or a pregnancy which had ended in miscarriage), spontaneously or after fertility treatment, than younger men (≤35 years, Group I).

Overall DFI was 17.3 ± 10.9% (range 0.5–66). Mean DFI was 15.7 ± 10.2% (range 0.5–66) in Group I, 18.2 ± 11.3% (range 1–60.5) in Group II, and 18.3 ± 11 (range 1.5–58) in Group III. DFI was significantly lower (by Mann–Whitney *U* test) in Group I than Group II ($P = 0.034$) or III ($P = 0.022$). There was no difference in DFI between Group II and III. **Figure 1** summarizes this result. Also, regression analysis demonstrated a significant increase in sperm DFI with age ($P = 0.02$; Spearman's rank correlation coefficient, $r = 0.10$). **Figure 2** summarizes this result.

Discussion

These results demonstrate that sperm DNA-strand damage measured by TUNEL assay significantly increases ($P = 0.02$) with subject age in a clinically large sample of men undergoing infertility treatment or investigation. The findings are in contrast to Sun *et al*. (1997), who did not find any relationship between DNA damage and age after measuring sperm DNA fragmentation by TUNEL in samples of 291 subjects in an infertility programme. However, besides sample size being different from this study, they analysed DNA damage after swim-up sperm wash, a procedure that may significantly decrease the proportion of chromatin-fragmented spermatozoa

Table 1. General characteristics of the study population.

Characteristic	*Occurrence*
Patients (*n*)	508
Age in years (mean ± SD; range)	37.7 ± 6.2; 22–69
Fathered at least one child	24.6 (125)
Abstinence (days) (mean ± SD)	3.6 ± 2.3
Semen samples[a]	
Normozoospermia	59.0 (300)
Oligozoospermia	6.9 (35)
Asthenozoospermia	6.5 (33)
Teratozoospermia	10.6 (54)
Oligoasthenozoospermia	1.8 (9)
Oligoteratozoospermia	1.4 (7)
Asthenoteratozoospermia	9.3 (47)
Oligoasthenoteratozoospermia	4.5 (23)
Leukocytes in semen ($x10^6$) (mean ± SD)	0.5 ± 0.9
Varicocele	
Yes	20.9 (106)
No	79.1 (402)
Tobacco use	
Yes	12.2 (62)
No	87.8 (446)
Regular alcohol use	
Yes	49.2 (250)
No	50.8 (258)
Vitamin supplement use	
Yes	24.8 (126)
No	75.2 (382)

Values are percentages (number) unless otherwise stated.
[a]Categorized according to World Health Organization (1999).

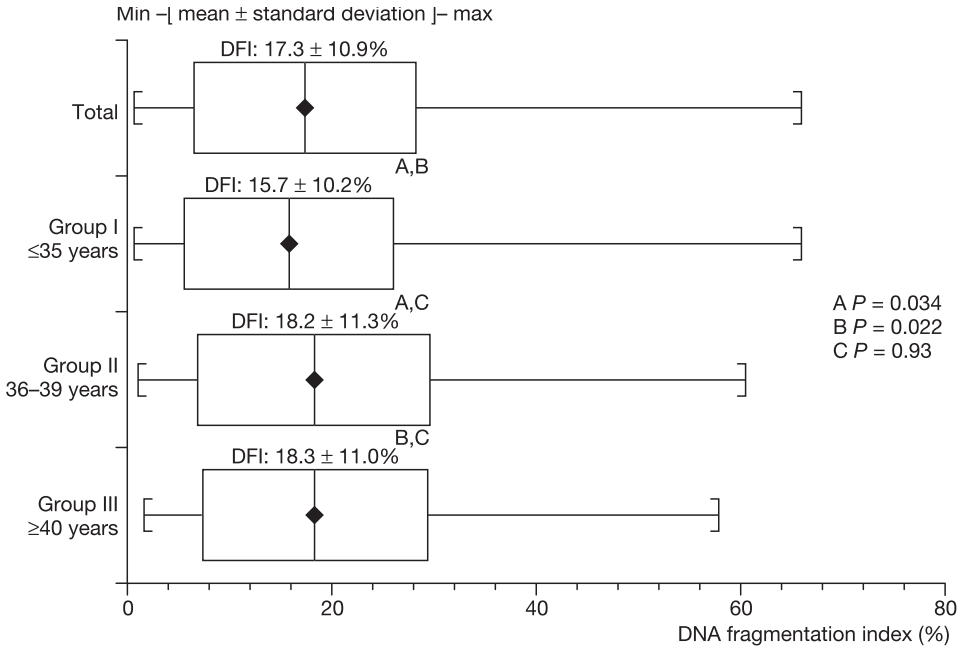

Figure 1. DNA fragmentation index (DFI) according to age: three age cut-off points are compared. Percentage of sperm with highly fragmented DNA in the older groups II and III is significantly greater than that in the younger group I (A: $P = 0.034$; B: $P = 0.022$, Mann–Whitney U test). There was no difference in DFI between older groups (C: $P = 0.93$, Mann–Whitney U test).

Table 2. General characteristics of the three age groups studied.

Characteristic	*Group I (≤35 years)*	*Group II (36–39 years)*	*Group III (≥40 years)*
Patients (*n*)	186	140	182
Age in years (mean ± SD)	31.7 ± 2.7	37.3 ± 1.1	44.2 ± 4.5
Fathered at least one child	17.2[a] (32)	25.0 (35)	31.9[a] (58)
Abstinence (days) (mean ± SD)	3.6 ± 2.3	3.5 ± 2.4	3.5 ± 2.3
Semen samples[b]			
Normozoospermia	60.2 (112)	58.6 (82)	58.2 (106)
Oligozoospermia	8.1 (15)	4.3 (6)	7.7 (14)
Asthenozoospermia	5.4 (10)	5.7 (8)	8.3 (15)
Teratozoospermia	9.7 (18)	15 (21)	8.3 (15)
Oligoasthenozoospermia	1.1 (2)	2.1 (3)	2.2 (4)
Oligoteratozoospermia	0.5 (1)	2.1 (3)	1.6 (3)
Asthenoteratozoospermia	8.6 (16)	7.1 (10)	11.5 (21)
Oligoasthenoteratozoospermia	6.4 (12)	5.0 (7)	2.2 (4)
Leukocytes in semen ($x10^6$) (mean ± SD)	0.5 ± 1.0	0.4 ± 0.6	0.6 ± 0.9
Varicocele			
Yes	18.3 (34)	25.0 (35)	20.3 (37)
No	81.7 (152)	75.0 (105)	79.7 (145)
Tobacco use			
Yes	10.2 (19)	14.3 (20)	12.6 (23)
No	89.8 (167)	85.7 (120)	87.4 (159)
Regular alcohol use			
Yes	46.8 (87)	51.4 (72)	50.0 (91)
No	53.2 (99)	48.6 (68)	50.0 (91)
Vitamin supplement use			
Yes	19.4 (36)	28.6 (40)	27.5 (50)
No	80.6 (150)	71.4 (100)	72.5 (132)

Values are percentages (number) unless otherwise stated.
[a]A significantly higher number of men in group III had fathered at least one child compared to group I ($P = 0.001$). There were no other significant differences between the groups.
[b]Categorized according to World Health Organization (1999).

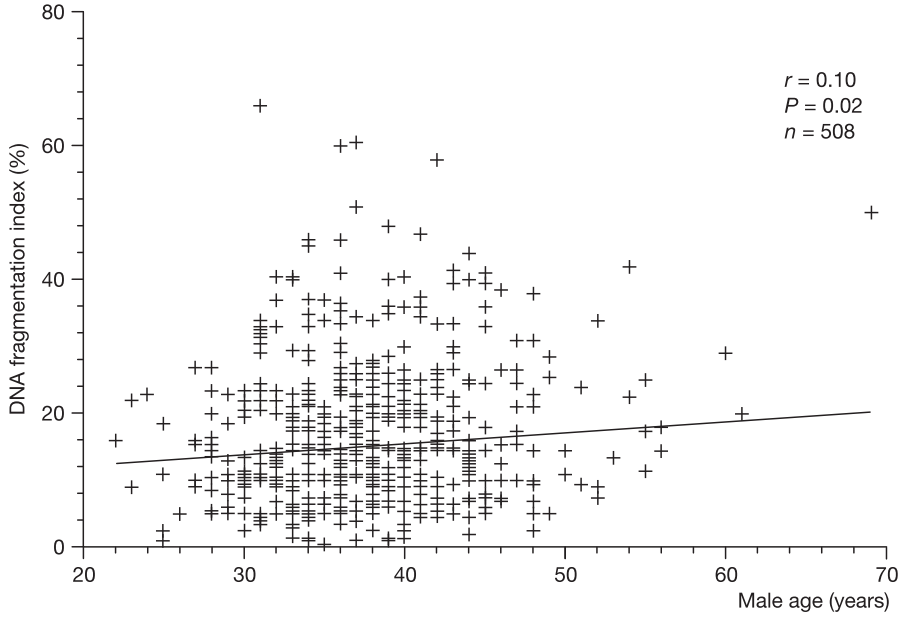

Figure 2. Relationship between male age (years) and sperm DNA damage. Individual data points and the regression line are shown. Spearman rank correlation coefficient = 0.1; $P = 0.02$.

(Piomboni *et al*., 2006) and therefore mask the true relationship between DNA damage and subject age. On the other hand, the findings in the present study are consistent with Morris *et al*. (2002), who studied 60 men participating in an IVF/intracytoplasmic sperm injection programme. They reported that sperm DNA damage measured by Comet assay under alkaline conditions was positively correlated with donor age. In addition, Moskovtsev *et al*. (2007) analysed DNA fragmentation by flow cytometry with acridine orange in sperm samples from 1230 unselected patients without azoospermia being seen for infertility evaluation and found it significantly correlated with patient's age.

Other authors have also found an association between sperm DNA damage and age in a non-infertile population. Using SCSA to analyse sperm samples from 277 men from an occupational hazard study with no known history of infertility, Spano *et al*. (1998) found a strong association between DFI and age. Wyrobek *et al*. (2006), also using SCSA, found a correlation between age and DNA damage by studying a group of 97 men. Using the Comet assay, Singh *et al*. (2003), who studied 66 men from an infertility clinic and a non-clinical group, found under neutral conditions, that increasing age correlated with increasing percentage of spermatozoa with highly damaged DNA. Schmid *et al*. (2007), using the Comet assay, found associations between male age and sperm DNA strand damage under alkaline conditions but not under neutral conditions in a non-clinical sample of active, healthy, non-smoking workers and retirees.

In this present analysis, significant changes in DNA damage were not observed for men of 36 years and older. Few other studies have performed the same kind of analysis but they have shown similar results. Moskovtsev *et al*. (2006) who divided 1125 patients into five age groups, observed that DFI was significantly increased in those over 45 years compared with all others age groups. Singh *et al*. (2003) demonstrated for three age breakpoints that the percentage of spermatozoa with highly damaged DNA was significantly greater in the older paired groups than the younger paired groups. Differences in the study populations, age breakpoints, and analysis method did not allow comparisons with this study's findings.

Despite the correlation between age and DFI being significant ($P = 0.02$), it could be considered weak (Spearman's $r = 0.10$). However, the correlation was quite similar to others found by different authors: Sun et al. (1997), $r = 0.06$, not significant; Schmid et al. (2007), $r = 0.22$, $P < 0.05$ for alkaline conditions and $r = 0.06$, $P = 0.58$ for neutral conditions; Moskovtsev et al. (2007), $r = 0.29$, $P < 0.001$. Other authors have reported stronger correlations: Singh et al. (2003) $r = 0.56$, $P < 0.001$; and Wyrobek et al. (2006), $r = 0.64$–0.72, $P < 0.001$. Differences in sample size, methods for evaluating DNA damage, and statistical analysis method probably contribute to the differences between these studies.

In conclusion, the results of this study clearly demonstrate an increase in sperm DNA damage with age in an infertile population measured by TUNEL assay. This age-related increase in sperm DNA damage predicts that older men may have increased risks of unsuccessful and abnormal pregnancies as a consequence of fertilization with damaged spermatozoa. This information may be useful in the medical management of infertile men.

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