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ORIGINAL ARTICLE *Andrology*

DNA fragmentation of sperm: a radical examination of the contribution of oxidative stress and age in 16 945 semen samples

D. A. Vaughan[1,2,3,†], E. Tirado[4,†,‡], D. Garcia[5], V. Datta[4], and D. Sakkas[3,*]

[1]Department of Obstetrics and Gynecology, Beth Israel Deaconess Medical Center, Boston, MA 02115, USA [2]Department of Obstetrics, Gynecology and Reproductive Biology, Harvard Medical School, Boston, MA 02115, USA [3]Boston IVF, Waltham, MA 02445, USA [4]ReproSource Fertility Diagnostics, Marlborough, MA 01752, USA [5]Clinica EUGIN, Barcelona 08029, Spain

*Correspondence address. Boston IVF, 130 2nd Ave, Waltham, MA 02445, USA. E-mail: dsakkas@bostonivf.com

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STUDY QUESTION: What is the relationship between sperm DNA fragmentation and oxidative stress (OS) with increasing male age?

SUMMARY ANSWER: Sperm DNA fragmentation increases with age and is likely related to both defective spermatogenesis and increasing OS levels.

WHAT IS KNOWN ALREADY: Sperm quality declines with age. The presence of DNA damage in a high fraction of spermatozoa from a raw semen sample is associated with lower male fertility in natural conception and intrauterine insemination.

STUDY DESIGN, SIZE, DURATION: A retrospective cohort study of 16 945 semen samples analysed at a single reference laboratory between January 2010 and December 2018.

PARTICIPANTS/MATERIALS, SETTING, METHODS: All males were undergoing an infertility evaluation. The cohort was divided into seven age categories: <30, 30–34, 35–39, 40–44, 45–49, 50 to <54 and ≥55 years. The mean age was 37.6 years (SD 6.8). Sperm DNA fragmentation index (DFI) and high DNA stainability (HDS) were calculated using flow cytometry. OS levels were measured using the oxidative stress adducts (OSA) test, by spectrophotometry. ANOVA with weighted polynomial contrast analysis was used to evaluate trends for DFI, OSA and HDS values across age categories.

MAIN RESULTS AND THE ROLE OF CHANCE: Mean DFI significantly increased across all age groups ($P_{trend} < 0.001$). OSA was lowest in patients <30 years old (mean 3.6, SD 1.0) and also increased as age increased ($P_{trend} < 0.001$). There was a statistically significant difference between age groups for each of the three parameters ($P < 0.001$). There was a significant linear trend for DFI, OSA and HDS across the seven age categories ($P < 0.001$). Among patients with high DFI, there was a decreasing age-dependent trend in the patients observed with high OSA ($P < 0.001$).

LIMITATIONS, REASONS FOR CAUTION: This is a retrospective study. All males included in the study were undergoing a work-up for infertility and may not be representative of a fertile population. Additional patient demographics and clinical data were not available.

WIDER IMPLICATIONS OF THE FINDINGS: DNA and/or oxidative damage in sperm may be just as important to understand as the chromosomal aberrations that are carried in the oocyte. Further studies are needed to evaluate the effect of advancing paternal age on the male genome and, ultimately, on the health of the offspring.

STUDY FUNDING/COMPETING INTEREST(S): No funding was obtained for this study. V.D. is an employee of Reprosource/Quest Diagnostics. D.S. reports he was a Scientific Advisor to Cooper Surgical.

TRIAL REGISTRATION NUMBER: N/A

Key words: male infertility / sperm quality / sperm DNA damage / sperm DNA fragmentation / oxidative stress

[†]The first two authors are co-first authors.

[‡]This paper is dedicated to the memory of our dear colleague, Edna Tirado, PhD, who passed away during the preparation of this manuscript.

Introduction

Male infertility is thought to be the primary or a contributory factor in up to 50% of couples presenting with infertility (World Health Organization, 1992). In 1992, the report of a successful pregnancy using ICSI (Palermo *et al.*, 1992) transformed the ability to care for infertile males and opened up new avenues to treat the majority of males, irrespective of subfertility aetiology. These remarkable advances, however, have led to fears that the quality of the paternal genome being introduced into the egg may be compromised (Lewis and Kumar, 2015). Despite taking the growing importance of the paternal genome into account, sperm is still perceived as a minor factor when evaluating the infertile couple. Current clinical practice often only involves a brief medical history and a single semen analysis (Practice Committee of the American Society for Reproductive Medicine, 2015).

In animal studies, an abnormal paternal component, particularly the sperm DNA and chromatin, have been demonstrated to have adverse effects on reproductive outcomes and progeny (Hales *et al.*, 1992; Robaire and Hales, 2003; Anway *et al.*, 2005; Miska and Ferguson-Smith, 2016). Forty years ago, Evenson *et al.* (1980) first showed a relationship between sperm chromatin heterogeneity and fertility in bulls, mice and humans. While there is a more definitive interrelationship between sperm chromatin/DNA damage and reproductive outcome in animal models compared to the human, the contemporary data on human sperm DNA testing would suggest that: (i) the probability of fathering a child, by either natural conception or IUI is reduced if the raw semen sample contains a high fraction of spermatozoa with DNA damage, regardless of semen analysis parameters (Bungum *et al.*, 2007; Frydman *et al.*, 2008; Zini *et al.*, 2008; Bungum *et al.*, 2011; Oleszczuk *et al.*, 2016), (ii) IUI attempts should be reduced and IVF/ICSI favoured when the initial evaluation of the male indicates an increased sperm DNA fragmentation (Zhao *et al.*, 2014). The clinical application of the numerous commercially available sperm DNA damage tests is still controversial (Collins *et al.*, 2008; Simon *et al.*, 2014; Agarwal *et al.*, 2016); however, it is unquestionable that infertile men or men from infertile couples have a higher proportion of DNA damage in sperm than fertile controls (Zini *et al.*, 2001; Wiweko and Utami, 2017). In addition, there is increasing evidence that sperm DNA damage abnormalities in men are associated with higher miscarriage rates (Ribas-Maynou *et al.*, 2012; Robinson *et al.*, 2012; Ruixue *et al.*, 2013) and that selection techniques that maximize the chance of selecting sperm with good DNA integrity may in fact reduce the incidence of miscarriage (Lepine *et al.*, 2019; Miller *et al.*, 2019; Parrella *et al.*, 2019).

The pathogenesis behind chromatin and DNA anomalies in ejaculated sperm remains poorly defined. Several mechanisms have been postulated including: abnormalities in chromatin dynamics and remodelling during spermiogenesis; apoptosis in the seminiferous tubules; DNA fragmentation induced mainly by oxygen radicals, including the hydroxyl radical and nitric oxide, during sperm transport damage induced by external agents, such as chemotherapy, radiotherapy or environmental toxicants (Sakkas and Alvarez, 2010; Aitken and Curry, 2011).

One of the major mechanisms believed to relate to sperm DNA integrity is oxidative stress (OS). Elevated OS levels are present in 30–80% of infertile men and represent a common mediator between various disease conditions and impaired reproductive potential (Agarwal *et al.*, 2006). OS is a well-recognized mechanism that plays an important role in the aetiology of male infertility and results from an imbalance between reactive oxygen species (ROS) and antioxidant capacity (Aitken *et al.*, 2012; Elbardisi *et al.*, 2019; Agarwal *et al.*, 2019; Aitken, 2020). The human sperm plasma membrane is extremely sensitive to attack by OS because it contains high concentrations of polyunsaturated fatty acids (PUFAs). These PUFAs control the plasma membrane fluidity and physiological levels of ROS. This is required to provide the biochemical precursors required to sustain important biochemical and biological sperm functions, such as maintenance of ATPase activity; hyperactivation, capacitation, membrane fusion events associated with the acrosome reaction and union with the oocyte (de Lamirande and Gagnon, 1993; Aitken *et al.*, 1997; Ochsendorf *et al.*, 1998).

OS can induce damage to sperm cellular structures, including the sperm plasma membrane, initiating a lipid peroxidation (LPO) cascade that results in loss of plasma membrane integrity properties and other functions of the plasma membrane causing defective sperm function and a corresponding decrease in sperm fertilization capacity (Singer *et al.*, 1982; Esterbauer *et al.*, 1991; Alleva *et al.*, 1997; Zini *et al.*, 2000). The clinical significance of the end products of LPO can be seen in the correlations consistently observed between peroxidative damage and decreased sperm function including: changes in the sperm membrane permeability; decreases in sperm motility; premature acrosome reaction; diminished capacity to interact and penetrate an oocyte; apoptosis and DNA fragmentation (Nakamura *et al.*, 2002; Koca *et al.*, 2003; Moustafa *et al.*, 2004). Overarching the issue of the quality of the paternal genome is the impact of ageing. It is now very clear that sperm quality declines with age (Wyrobek *et al.*, 2006; Plastira *et al.*, 2007; Humm and Sakkas, 2013).

The relationship between age, OS and nuclear DNA integrity in sperm is, however, still not well defined in male infertility patients. A key question is whether patients with sperm DNA damage always have concurrent OS in their sperm. The aim of this study was to evaluate, in the same semen sample, sperm DNA damage and LPO damage of the sperm membrane from almost 17 000 males being evaluated for infertility.

Materials and methods

Study samples

DNA fragmentation index (DFI), high DNA stainability (HDS) and oxidative stress adducts (OSA) values were measured on all samples. Samples were collected between January 2010 and December 2018. A total of 16 945 samples were available from analysis. Over 98% of these samples were from unique patients with the remainder being repeat visits.

Semen analysis procedure

The semen sample was collected after 3–5 days' abstinence by masturbation. Thirty minutes following collection, semen samples collected were equally aliquoted and transferred into two 2 ml cryovials; the frozen raw semen samples were then shipped on dry ice to the reference laboratory.

For the sperm DNA fragmentation assay, a sperm solution of 2×10^6 per ml was prepared based on the sample's initial sperm concentration and an aliquot of thawed semen was diluted in TNE buffer (0.15 M NaCl, 0.01 M Tris-HCl, 0.001 M EDTA; pH 7.4). The remaining sample was used to perform the OSA test.

Sperm DNA fragmentation test

The sperm DNA fragmentation assay measures both the DFI and HDS (%) and is based on the original method developed by Evenson *et al.* (1991). DFI assay (DFI%) represents the percentage of cells with damaged DNA compared to the total number of sperm. The degree of DNA denaturation is measured by intercalation of Acridine Orange after acid treatment using FC-500 Beckman Coulter Flow cytometer. A sperm solution of 2×10^6 per ml was subjected to 0.1N HCL , Acridine Orange stains normal double-stranded DNA green, and denatured, single-stranded DNA is stained red. The DFI, which is the amount of red fluorescence divided by the sum of red and green fluorescence, is an approximate measure of the proportion of single stranded to total DNA in each sperm.

The DFI assay also detects a percent of spermatozoa with a more *rounded* head in a sample, which is represented by the HDS, indicating the proportion of immature sperm with defects in the histone-to-protamine transition. These *rounded* sperm emit higher amounts of green fluorescence because they have less chromatin condensation (Plastira *et al.*, 2007).

Oxidative stress adducts test

LPO is a well-established mechanism of cellular injury. Lipid peroxides are highly unstable and, in the presence of ROS decompose to form a complex series of compounds including reactive carbonyl compounds. One example of this type of reaction is the formation of malondialdehyde and lipid aldehydes when PUFA peroxides react with ROS. The levels of OS in the remaining fraction of the sample were measured by the OSA test, which is a spectrophotometric method, capable of detecting the production of adducts produced during OS. The test is based on the method previously described by Gomez *et al.* (1998). A previous clinical assessment of 955 men from infertile couples compared with 20 fertile controls showed that an abnormal OSA result highly correlated with infertility ($P < 0.05$) and showed that the cut-offs of <3.8, 3.8–4.4 and >4.4 were clinically relevant (Tirado *et al.*, 2010).

Statistical analysis

Data were analysed to understand the inter-relationships between all parameters measured.

The following assessments were performed on each semen sample: % DFI, % HDS and OSA-μM. The cohort was divided into seven age categories: <30, 30–34, 35–39, 40–44, 45–49, 50-54, ≥55 years.

Categories of DFI and OSA were examined to understand their inter-relationship when each parameter was low (L), medium (M) or high (H). These categories were defined as follows for DFI: <20, 20–30 and >30, and for OSA: <3.8, 3.8 to <4.4 and ≥4.4 (Fig. 1).

ANOVA with weighted polynomial contrast analysis was used to evaluate trends for DFI, OSA and HDS values across age categories, using SPSS software version 22.0 (Armonk, NY, USA). A *P*-value <0.05 was set as statistically significant.

The study was deemed exempt by the New England Independent Review Board.

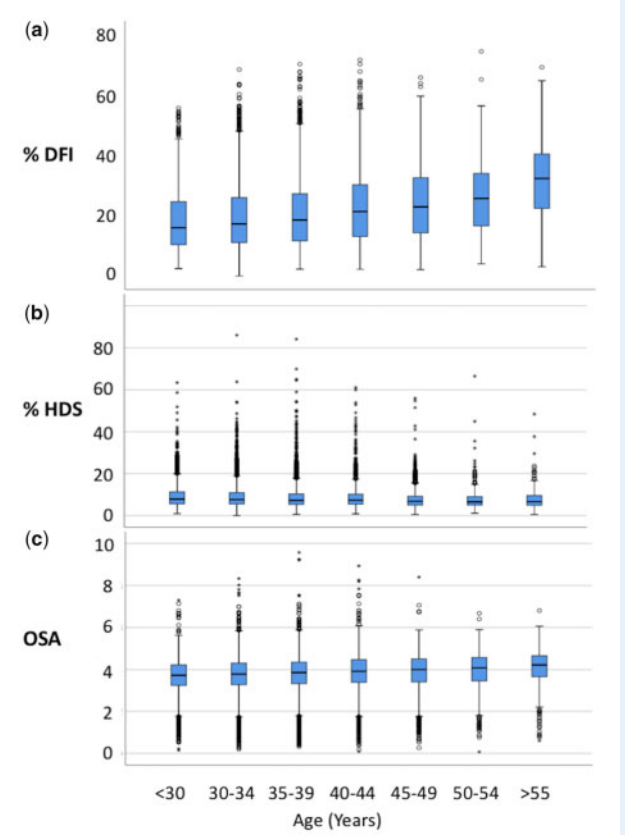

Figure 1. A box plot with outliers showing (a) DNA fragmentation index (% DFI), (b) high density stainability (% HDS) and (c) oxidative stress adducts (OSA) by age categories. Individual samples are represented by '*' and 'o'

Results

There were a total of 16 945 samples available for analysis. Cohort OSA, DFI and HDS characteristics are shown in Table I.

Table II illustrates the cohort distribution and sperm characteristics according to each of the seven age categories outlined previously. Mean DFI was lowest in the youngest age group (<30 years, mean DFI 18.2%, SD 10.1) and significantly increased across all age groups ($P_{trend} < 0.001$). It was highest in the oldest age group (≥55 years, mean 30.1%, SD 13.6). Of note are the outliers present in the younger age groups when examining %DFI. Figure 1 indicates the variability outside the upper quartile, which could infer a proportion of patients showing clinical differences from the median population of patients assessed. Similarly, OSA was lowest in patients <30 years old (mean 3.6, SD 1.0) and increased as age increased ($P_{trend} < 0.001$). HDS was

highest in the youngest age group (mean 9.4, SD 6.0) and decreased across the age groups ($P_{trend} < 0.001$).

We examined the relationship between age categories and DFI, OSA and HDS using a one-sided ANOVA. There was a statistically significant difference between age groups for each of the three parameters ($P < 0.001$, see Table II).

To test if there was a linear trend across age categories, an ANOVA with polynomic contrast was performed. Again, there was a significant linear trend for DFI, OSA and HDS across the seven age categories ($P < 0.001$). There was a significant quadratic relationship for DFI across age categories ($P < 0.001$, see Table II) but not for OSA and HDS.

DFI and OSA combined

In total, 6163/8550 (72.1%) patients with low DFI also had low levels of OSA. Similarly, 2974/3818 (77.9%) of those with a DFI >30 also had high OSA. Figure 2 is a set of pie charts depicting the relationship between age, OSA and DFI (see also Supplementary Fig. S1).

A subanalysis was performed in the high DFI group to further understand the relationship between age and OSA. This analysis revealed that among patients with high DFI, there was a decreasing age-dependent trend in the patients observed with high OSA (Cochran–Armitage Trend test: n = 3806, $Z = 3.7$, $P < 0.001$). The percentage of high OSA patients in the high DFI group decreased by age, from 76% in the <30-year-old group to 69% in those ≥50 years.

Table I The number of samples (n), range, overall mean and SD of all samples in relation to age, oxidative stress adducts (OSA) measurement, DNA fragmentation index (DFI) and high DNA stainability (HDS).

	n	Min	Max	Mean	SD
Age	16 945	18.86	86.84	37.60	6.84
OSA	16 945	0.07	18.80	3.75	0.98
DFI (%)	16 945	0	74.36	21.05	11.18
HDS (%)	16 945	0	86.03	8.76	5.55

Discussion

This study shows a remarkable correlation between age and the DFI of sperm in almost 17 000 samples. The strong association between DFI and age validates the work of others (Wyrobek *et al.*, 2006; Plastira *et al.*, 2007). Furthermore, the lipid oxidation status of the sperm membrane also shows increasing OSA values with age and increasing DFI. Interestingly, our study suggests that younger patients with high sperm DFI are more likely to be related to OS than those in older age groups.

Male age at conception has been rising in parallel with increasing female age (Khandwala *et al.*, 2017), which is of concern as there is an increasing body of evidence that associates increasing paternal age at conception with a number of conditions including psychiatric disorders such as schizophrenia and autism as well as a possible increase in birth defects (Humm and Sakkas, 2013; Oldereid *et al.*, 2018). D'Onofrio *et al.* (2014) have also described an association between paternal age at childbearing and offspring psychiatric and academic morbidity. There is some suggestion that there may be confounders in the population of older fathers and that they may represent a somewhat atypical group. For example, both higher and lower socioeconomic statuses are over-represented in the group (Nilsen *et al.*, 2013). Some studies of autism suggest that even advanced grand paternal age (the age at which the grandfather conceived the father of the child in question) may be a risk factor for autism (Frans *et al.*, 2013). The underlying reasons are difficult to assess although more recent studies are now focusing on mutation rates in particular. Of greatest concern are points raised by two key papers. One proposed an exponential model estimating that paternal mutations double every 16.5 years (Kong *et al.*, 2012) and a second, more recent study, demonstrating the relationship between sperm mosaicism and autism spectrum disorders in offspring (Breuss *et al.*, 2020).

With respect to the literature on sperm DNA fragmentation with pregnancy outcomes, both the terminal deoxynucleotidyl transferase-mediated dUDP nick-end labelling and Comet assays have been used to measure sperm from recurrent pregnancy loss (RPL) couples and have shown that the male partners of women with high rates of RPL have increased levels of DNA damage (Carrell *et al.*, 2003; Ribas-Maynou *et al.*, 2012). Bungum *et al.* (2007) have also shown that the odds ratio for women achieving a pregnancy after intra-uterine

Table II The number of samples (n), mean and SD for DFI, OSA measurement and HDS when comparing age subgroups.

Age (years)	n	DFI % mean (SD)	OSA μM mean (SD)	HDS % mean (SD)
<30	1471	18.2 (10.1)	3.6 (1.0)	9.4 (6.0)
30–34	4633	19.6 (10.5)	3.7 (1.0)	9.2 (5.8)
35–39	5122	20.4 (10.8)	3.7 (1.0)	8.7 (5.5)
40–44	3255	22.2 (11.2)	3.8 (1.0)	8.6 (5.3)
45–49	1544	23.6 (11.8)	3.9 (1.0)	8.1 (5.0)
50–54	567	26.5 (11.9)	3.9 (1.0)	7.9 (5.2)
≥55 years	354	30.1 (13.6)	4.0 (1.0)	7.8 (4.9)
Weighted Linear trend *P*-value		<0.001	<0.001	<0.001
Weighted quadratic trend *P*-value		<0.001	0.78	0.49

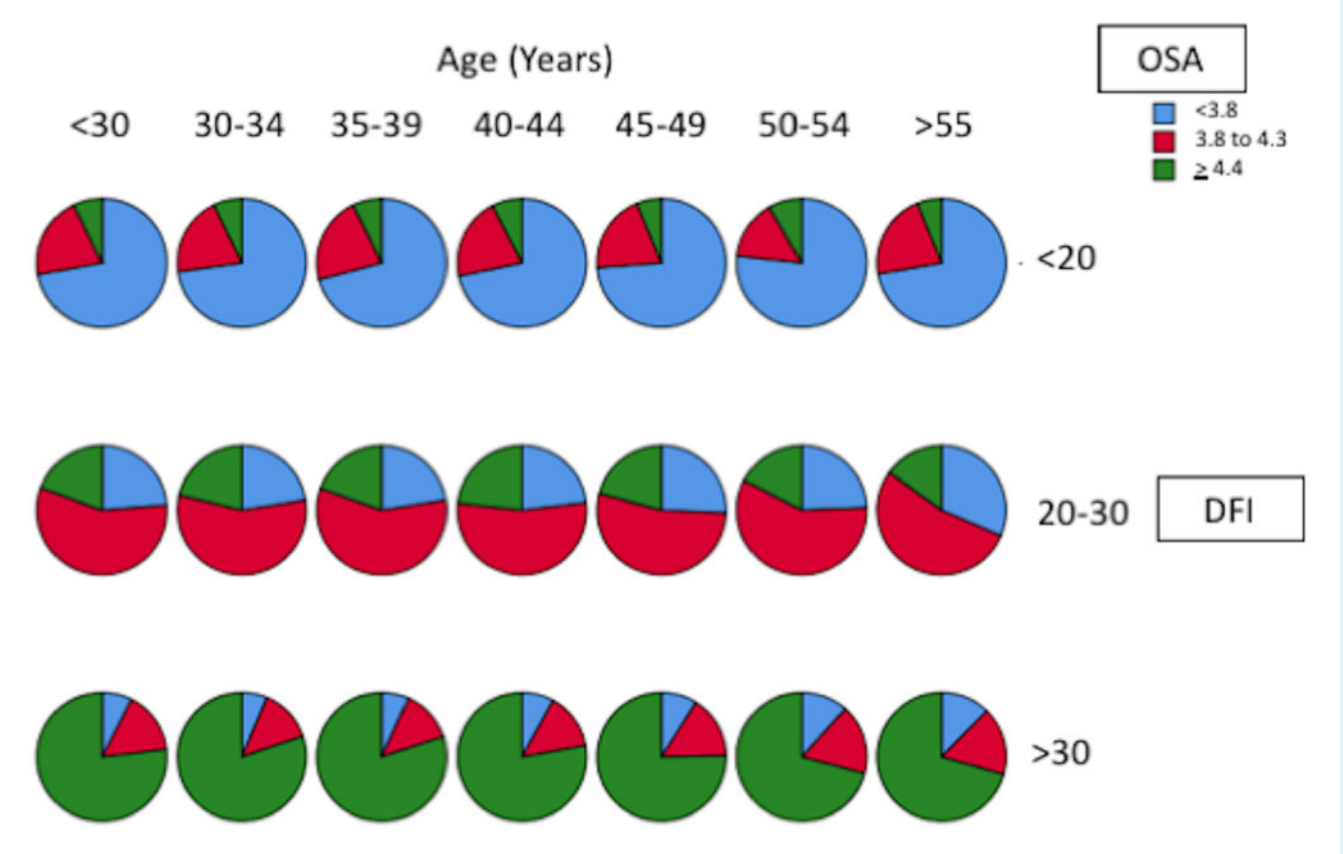

Figure 2. Pie chart depiction of DFI and OSA across age categories.

insemination treatment is drastically reduced when the male partner has high (>30%) levels of sperm nuclear DNA damage as assessed by the sperm chromatin structure assay.

In the present study, sperm membrane damage as measured by the OSA assessment also showed an increase with male age. Patients with a DFI of greater than 30% however also showed a dramatic increase in their OSA values. Interestingly, sperm DNA damage is thought to be largely oxidative and to be closely associated with defects in spermiogenesis. Aitken and Curry (2011) have postulated that spermiogenesis is disrupted by OS, leading to the creation of defective gametes with poorly remodelled chromatin that are particularly susceptible to free radical attack. It can also be argued that faulty chromatin remodelling during spermiogenesis is a primary cause of sperm DNA damage and further exacerbates the susceptibility of sperm DNA to oxidative attack. Our data indicate that sperm membrane oxidative damage definitely increases as sperm DNA damage increases, however, a significant population of sperm exists in lower DFI patients with medium and high oxidative membrane stress (Fig. 1). The pie charts also indicate that in many patients the two do not always occur concurrently (Fig. 2). Overall this could indicate that there are two populations of DNA damaged sperm; (i) the ones that originate from spermiogenesis with some DNA damage and (ii) those that are processed correctly during spermatogenesis but are subjected to OS leading to an acquisition of or increase in DNA damage during transport in the reproductive tract (Sakkas and Alvarez, 2010). The present study found higher levels of HDS amongst younger patients. There is evidence that the HDS represents immature forms of spermatozoa which are not fully condensed so that acridine orange can penetrate to the double-stranded DNA within (Zini *et al.*, 2009). In relation to HDS, Evenson *et al.* (1999) performed a study in which 82% of the couples conceived within 12 months without undergoing assisted reproduction procedures. The time to achieving pregnancy was recorded and they found that the mean HDS of 73 couples achieving pregnancy within 3 months was 8.95%, while the mean HDS of 31 couples who did not achieve pregnancy within 12 months was far higher (15.03%, $P < 0.001$).

The literature is mixed regarding the correlation between HDS and age (Boe-Hansen *et al.*, 2006; Lin *et al.*, 2008; Deenadayal Mettler *et al.*, 2019). This observation may be explained by a higher leukocyte presence in the ejaculate of younger males however, it is also likely to occur from different processes. When we examined the subset of patients with high DFI across age groups, we noted that OSA in patients <30 and 30–34 was 77% and 80%, respectively. In contrast in older patients, the relative contribution of OSA to high DFI was less (69% in those ≥50 years), suggesting that defective spermiogenesis plays more of a role in this group. This is in keeping with an interesting study by Muratori *et al.* (2015) that suggested that oxidative attack occurs following spermiation, in susceptible cells and that it occurs

mainly in live sperm. The prevalence of samples with medium or high OSA and medium DFI levels may also indicate that these sperm may be in the process of developing DNA fragmentation. Mitchell *et al.* (2011) have previously demonstrated that sperm DNA fragmentation is higher in unviable sperm, and this may explain the elevated DFI seen in the older age categories in the present study. The underlying mechanisms that drive either DNA damage or OS may still accumulate with age, exacerbating the situation. The complex relationship existing between OS and sperm nuclear DNA damage has been extensively reviewed in previous studies and it is still unclear which comes first: the chicken (OS) or the egg (sperm DNA damage) (Bui *et al.*, 2018; Drevet and Aitken, 2019; Aitken, 2020). One area of interest is how DNA damage is dealt with during spermatogenesis and how this changes with age. For example, we have previously shown significant differences in DNA damage repair-associated proteins (Poly [ADP-ribose] polymerase-1 (PARP-1), Protease-activated receptors (PAR), X-ray repair cross-complementing protein-1 (XRCC1) and Apurinic/apyrimidinic Endonuclease-1 (APE1)), and apoptosis markers (caspase 9, active caspase 3 and cleaved PARP-1) in testicular samples from older men, and in particular during the spermatocyte stage (El-Domyati *et al.*, 2009).

Strengths and limitations

The primary strength of this study lies in the large numbers and concurrent analysis of DFI, HDS and OSA from the same semen sample. This is one of the largest studies to date to examine the relationship between age, DFI, OSA and HDS. As a result, we could examine the inter-relationships in detail. One limitation of this study is that all males included in the study were undergoing a work-up for infertility and therefore it is likely not representative of the general, fertile population. A comparative cohort of fertile men would better assist us in evaluating the overall role of the male genome and, ultimately, on the health of the offspring. A second limitation is that additional patient demographics and clinical data, including the patient's semen analysis and infertility aetiology, were not available.

Conclusions

This article highlights, in a large cohort of males, that sperm quality reflected by sperm DNA fragmentation and oxidative status declines with age. The clinical implications of this study highlight the impact of age on sperm quality in a large series of patients. In the female, the decline in reproductive efficiency is more drastic, in particular when taking into account the higher levels of aneuploidy in the egg and embryos and the chance of aneuploidy in the conceptus once women are over 40 (Hook, 1976). Much effort has been made to assess aneuploidy of the embryo during IVF (Wells *et al.*, 2008), however more comprehensive sperm testing is largely ignored and in many cases deemed not necessary (Collins *et al.*, 2008; Barratt *et al.*, 2017). It has been previously argued that even though assessment of DNA damage may not correlate directly with some aspects of IVF outcomes it may still reveal male-related effects on the developmental normality of the embryo and the health of possible future children (Seli and Sakkas, 2005; Lewis *et al.*, 2008; Aitken *et al.*, 2013; Humm and Sakkas, 2013). In males, the presence of both sperm DNA and oxidative damage may only be one part of a cascade of events indicative of subtler abnormalities, including mutations and epigenetic modifications. We believe that this is an area that warrants further investigation and longitudinal studies.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Edna Tirado PhD, sadly passed away during the preparation of this manuscript. We would like to recognize Edna's passion for sperm biology and without whom, this study would not have been possible.

Authors' roles

D.A.V., E.T. and D.S. were responsible for the study concept and design. D.G. performed and advised on the statistical analyses. D.A.V, E.T., V.D. and D.S. interpreted the data. All authors were responsible for the writing and editing of the article and all participated in critical revision and final approval of the article.

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Conflict of interest

V.D. is an employee of Reprosource/Quest Diagnostics. D.S. reports he was a Scientific Advisor to Cooper Surgical.

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