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ARTICLE

Age-related changes in human conventional semen parameters and sperm chromatin structure assay-defined sperm DNA/chromatin integrity

BIOGRAPHY

Jiangman Gao gained her Master of Medicine in 2013. She is now a PhD candidate at the Center for Reproductive Medicine, Department of Obstetrics and Gynecology, Peking University Third Hospital, under the guidance of Professor Jie Qiao. The focus of her research is on male infertility and sperm DNA/chromatin damage.

Jiangman Gao[1,3,4], Renpei Yuan[1], Siwei Yang[1], Yuanyuan Wang[1,3,4], Ying Huang[1], Liying Yan[1,2,3,4], Hui Jiang[1,*], Jie Qiao[1,2,3,4,*]

KEY MESSAGE

Age was negatively correlated with semen volume, total sperm count, motility and HDS, and positively with sperm concentration and DFI. Routine sperm parameters have significant correlations with sperm DFI and HDS. This study highlighted the impact of age on sperm quality and reinforced that both DFI and HDS evaluation may play a role in assessing potential male infertility.

ABSTRACT

Research question: What are the correlations between male age, traditional semen parameters, sperm DNA fragmentation index (DFI) and high DNA stainability (HDS) in a sufficiently large sample size?

Design: Retrospective cohort study of 18,441 semen samples, with data divided into seven age groups according to male age: ≤25, 26–30, 31–35, 36–40, 41–45, 46–50 and ≥51 years.

Results: Age was negatively correlated with semen volume, total sperm count, motility and HDS, and positively correlated with sperm concentration and DFI ($P < 0.001$). After 35 years of age, semen volume and total sperm count began to decline. After 30 years of age, motility and HDS decreased consistently. Sperm concentration and DFI increased from 26–30 years of age. DFI was negatively correlated with sperm concentration, total sperm count, motility and normal morphology ($P < 0.001$) and positively correlated with semen volume and HDS ($P < 0.001$). HDS was negatively correlated with all parameters ($P < 0.001$) except semen volume ($r = -0.013$, $P = 0.074$) and DFI ($r = 0.124$, $P < 0.001$). Patients aged ≥40 years had higher DFI than those aged <40 years in the entire cohort, in the abnormal semen parameters cohort, and in the normal semen parameters cohort (OR 2.145, 2.042, 1.948, respectively, $P < 0.001$). The ≥40 years age group had a lower HDS than the <40 years age group in the entire cohort and abnormal semen parameters cohort (OR 0.719, 0.677, respectively, $P < 0.001$).

Conclusions: Ageing is a negative effector of sperm quantity and quality, and routine sperm parameters have weak but significant correlations with sperm DNA/chromatin integrity.

[1] Center for Reproductive Medicine, Department of Obstetrics and Gynecology, Peking University Third Hospital, Beijing 100191, China
[2] National Clinical Research Center for Obstetrics and Gynecology, Peking University Third Hospital, Beijing 100191, China
[3] Key Laboratory of Assisted Reproduction, Peking University, Ministry of Education, Beijing 100191, China
[4] Beijing Key Laboratory of Reproductive Endocrinology and Assisted Reproductive Technology, Peking University Third Hospital, Beijing 100191, China

*Corresponding authors. E-mail addresses: jianghui55@163.com (H. Jiang), jie.qiao@263.net (J. Qiao). https://doi.org/10.1016/j.rbmo.2021.02.006 1472-6483/
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KEYWORDS

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Sperm high DNA stainability

INTRODUCTION

In recent decades, the average age at first reproduction has risen significantly in many countries (*Humm and Sakkas, 2013*; *Khandwala et al., 2017*; *Schmidt et al., 2012*). Data are emerging to show that advanced parental age is associated with declines in fertility and offspring fitness. The effects of increased maternal age have been widely studied: it negatively influences the quantity and quality of oocytes and increases the likelihood of aneuploidies during embryo development (*Fragouli et al., 2006*; *Franasiak et al., 2014*; *Kuliev et al., 2003*). Comparatively, advanced paternal age is associated with a longer time to pregnancy and lower fertilization rates, embryo quality, implantation rates, pregnancy rates and live-born delivery rates (*Aboulghar et al., 2007*; *Bellver et al., 2008*; *Ferreira et al., 2010*; *Spandorfer et al., 1998*). It has a detrimental effect on pregnancy rates in cycles of conventional IVF (*Girsh et al., 2008*; *Horta et al., 2019*; *Kaarouch et al., 2018*; *Klonoff-Cohen and Natarajan, 2004*; *Sharma et al., 2015*), especially when the male is older than 40 years (*de La Rochebrochard et al., 2006*). Higher paternal age (45 years or older) is associated with an increased risk of premature birth, low birthweight and low Apgar score (*Khandwala et al., 2018*). Increased paternal age also probably increases potential maternal implications, such as pre-eclampsia and gestational diabetes (*Khandwala et al., 2017*). Advanced paternal age is also associated with a higher incidence of mental disorders among offspring, such as autism spectrum disorder and schizophrenia (*Conti and Eisenberg, 2016*; *Kimura et al., 2018*; *Sharma et al., 2015*), as well as a higher frequency of genetic disorders, such as Klinefelter syndrome, Down syndrome, Marfan syndrome and Apert's syndrome (*Lowe et al., 2001*; *Ramasamy et al., 2015*; *Wyrobek et al., 2006*).

Conventional semen analysis, which measures semen volume, sperm concentration, total sperm count, motility, progressive motility and morphology, has been widely used to study male fertility. Decreases in semen volume, total sperm count and motility are correlated with increasing paternal age (*Eskenazi et al., 2003*; *Guo et al., 2020*; *Hellstrom et al., 2006*; *Stone et al., 2013*). However, regarding sperm concentration, while some studies describe a decline with age (*Begueria et al., 2014*; *Stone et al., 2013*), others report no association (*Whitcomb et al., 2011*) or even an increase with age (*Rosiak-Gill et al., 2019*). Similarly, the reported effects of ageing on morphology are also inconsistent. Therefore, the conventional parameters of semen analysis are sometimes insufficient to evaluate the relationship between age and reproductive potential.

Currently, with the development of assisted reproductive technology (ART) and molecular biology, more indicators are arising to reflect sperm microstates, and one of these is sperm genetic integrity. In fact, sperm DNA damage might be a possible explanation for the negative effects of advanced male age on reproductive outcomes. To date, the literature relating to the effect of sperm DNA fragmentation on male infertility and ART treatment outcomes are conflicting, but it is still one of the most frequently debated topics in terms of the reasons for male infertility in reproductive medicine, especially in couples with unexplained infertility. Over the past few decades, multiple studies have evaluated the associations between sperm DNA fragmentation and male infertility, IVF, embryo development, clinical pregnancy, miscarriage and live birth, as summarized by *Simon et al. (2019)*. Nearly 100 articles relating to the effect of sperm DNA fragmentation on male infertility and the consequences of ART were included and reviewed. It was suggested that sperm DNA fragmentation is closely associated with male infertility and is independent of semen parameters. Sperm DNA fragmentation levels can influence ART outcomes. However, the clinical application of DNA fragmentation analysis is still controversial.

To determine sperm DNA damage, the sperm chromatin structure assay (SCSA), a high-precision flow cytometric test, is widely used in clinical settings. It can measure two sperm nuclear parameters simultaneously: sperm DNA strand breaks (% DNA fragmentation index [DFI]) and uncondensed chromatin (% high DNA stainability [HDS]) (*Evenson, 2017*). HDS is observed because acridine orange-stained histone-complexed DNA stains 2.3 times more than protamine-complexed DNA, and this HDS sperm fraction is easy to detect with the SCSA test (*Evenson et al., 1986*). DFI indicates the percentage of spermatozoa with fragmented DNA, and HDS measures the percentage of spermatozoa with an abnormal lack of chromatin condensation, proposed to be due to a suboptimal histone to protamine ratio. Whereas classical semen parameters vary widely within individuals for consecutive sampling, SCSA-defined DFI is an objective, biologically stable, sensitive and feasible measure of semen quality (*Evenson et al., 1991*), and it is a rapid, non-biased flow cytometer machine measurement providing robust statistical data with precision and repeatability in intra- and inter-laboratory results (*Evenson, 2016*). While previous studies have investigated the effect of advanced paternal age on sperm DNA damage, the results are inconsistent as different age groups and different assay techniques are used (*Brahem et al., 2011*; *Moskovtsev et al., 2006*; *Nijs et al., 2011*; *Simon et al., 2019*; *Vagnini et al., 2007*; *Varshini et al., 2012*).

Here, by obtaining a sufficiently large sample size of 18,441, the study laboratory was able to perform a valuable evaluation of the correlations of age with conventional semen parameters, DFI and HDS at the same time, to investigate male fertility. The aim of this study was to show the trends in conventional semen parameters, DFI and HDS with increasing age and to establish the correlations among HDS, DFI and conventional semen parameters. In addition, the aim was to look at sperm DNA damage more closely by comparing the prevalence of DFI (cut-off value 15%) of men ≥40 years to <40 years old in the entire cohort, in the subset with abnormal standard semen parameters, and in the subset with normal standard semen parameters.

MATERIALS AND METHODS

Subjects and semen analysis

This was a retrospective observational study of male patients who attended infertility clinics in the Andrology Laboratory of the Reproductive Medicine Center of Peking University Third Hospital from May 2018 to September 2019. The study was approved by the Institutional Review Board of Peking University Third Hospital (ref 2017SZ-048; approved 20 June 2017). A total of 18,441 samples from men aged 17–71 years were available for analysis. The data were divided into seven age categories: ≤25, 26–30, 31–35, 36–40, 41–45, 46–50

and ≥51 years. Two subgroups of men, ≥40 and <40 years, were also designated to assess the relationship between sperm DNA integrity and age.

Semen analysis was performed in the Andrology Laboratory using the same instruments. All semen samples were collected by masturbation after 2–7 days of abstinence and then analysed according to WHO guidelines after liquefaction at 37°C. After the semen volume was assessed, 10 µl sperm samples were placed into a Makler counting chamber (Sefi-Medical Instruments Ltd, Israel), and routine sperm inspection (concentration, progressive motility, non-progressive motility and immotility) was conducted by a computer-assisted sperm analysis system (SSA-II, Suijia Software Co. Ltd, Beijing, China). Normal sperm morphology was evaluated in samples stained under the modified Papanicolaou technique and analysed according to strict WHO criteria (World Health Organization, 2010). A minimum of 200 spermatozoa were examined in each sample. Sperm concentration, motility and morphology evaluations were assessed by rendering a total of 400 scored sperm cells. The total sperm count per ejaculate was calculated by multiplying the sperm concentration by the volume of semen in each sample. Motility was the sum of progressive and non-progressive motility.

SCSA test protocol

Determination of sperm DFI and HDS (%) was performed according to the method described in detail by *Evenson (2018)*. The SCSA kit was purchased from CellPro Biotech Co., Ltd (Ningbo, China). SCSA is based on staining sperm nuclei with acridine orange to evaluate the ratio of single- and double-stranded DNA. Briefly, an aliquot of raw semen was diluted with TNE Buffer (0.01 mol/l Tris HCl, 0.15 mol/l NaCl, 1 mmol/l EDTA) at 4°C to a final sperm concentration of 1–2 × 10^6 spermatozoa/ml. A volume of 200 µl of sperm suspension was mixed with 400 µl acid solution (0.1% Triton X-100, 0.15 mol/l NaCl and 0.08 N HCl, pH 1.20, 4°C) for 30 s, and 1.20 ml of acridine orange staining solution (6 µg/ml acridine orange, 370 ml of stock 0.1 mol/l citric acid, 630 ml of stock 0.2 mol/l Na_2HPO_4, 1 mmol/l disodium EDTA, 0.15 mol/l NaCl, pH 6.0, 4°C) was added, as previously described (*Evenson et al., 2020*). After 3 min of incubation, the sample was placed into a BD FACSCalibur™ flow cytometer (Becton Dickinson, San Jose, CA, USA) with the sample flowing to establish optimal sheath/sample flow. The fluorescence intensity of 5000 cells was recorded and analysed with data analysis software (DFIView 2010 Alpha 11.15, CellPro Biotech, Ningbo, China) for double-stranded (green: native) and single-stranded (red: damaged) DNA. The DFI was the ratio of red/red+green fluorescence, and HDS was the fraction of spermatozoa with a higher level of green fluorescence due to a lack of full exchange of histones for protamines. DNA damage is represented as percentage DFI, while immature spermatozoa with incomplete chromatin condensation are represented as percentage HDS.

Statistical analysis

All data analyses were conducted using SPSS Statistics for Windows, Version 26.0 (IBM Corp., Armonk, NY, USA). The results of semen analysis are expressed as the median (Q25, Q75) and mean ± SD. Non-parametric tests (Kruskal–Wallis single-factor analysis of variance) were used to determine the differences in semen parameters between groups. The growth rate of parameters of each age group relative to the preceding age group was calculated as follows: growth rate = (current age group – preceding younger age group)/younger age group. The correlations between parameters were examined using Pearson's correlation analysis. A chi-squared test was used to compare the categorical data. To define the risk of having high sperm DNA damage, OR were calculated and are presented with 95% CI and *P*-values. All statistical tests were two-sided, and *P*-values <0.05 were interpreted as statistically significant.

RESULTS

A total of 18,441 semen samples were included (age [mean ± SD] 34.00 ± 5.66 years; range 17–71 years) and divided into seven groups based on age: 483 patients were aged ≤25 years, 4818 patients were aged 26–30 years, 6920 patients were aged 31–35 years, 3946 patients were aged 36–40 years, 1509 patients were aged 41–45 years, 549 patients were aged 46–50 years, and 216 patients were aged ≥51 years. TABLE 1 shows the details of the male characteristics across age brackets.

Measured sperm parameters included semen volume, sperm concentration, total sperm count, total motility, progressive motility, normal morphology, DFI and HDS. The data are presented as medians (Q25, Q75) and mean ± SD. Among the age groups, semen volume, sperm concentration, total sperm count, total motility, progressive motility, DFI and HDS were significantly different ($P < 0.001$), as well as sperm morphology ($P = 0.012$) (TABLE 1). Multiple comparison statistical analysis showed that semen volume and total sperm count began to decline after 35 years of age, while total motility, progressive motility and HDS decreased consistently after 30 years of age. In contrast, sperm concentration and DFI increased significantly from younger to older groups (Supplementary Table 1).

FIGURE 1 shows the trends and Pearson's correlation among semen parameters and age. The averages of semen volume, total sperm count, total motility, progressive motility and HDS decreased from younger to older groups. They had a weak negative but significant correlation with age ($r = -0.138, -0.030, -0.119, -0.121, -0.076$, respectively, $P < 0.001$). Sperm concentration and DFI continued to increase from ≤25 years, and there was a weak positive but significant correlation with age ($r = 0.065$ and 0.225, respectively, $P < 0.001$). Although normal sperm morphology had a slightly positive correlation with age ($r = 0.020$, $P = 0.012$), the trend in average sperm morphology was not obvious. The total sperm count reached its peak in the group of patients aged 26–30 years. Overall, as age increased, semen volume, total motility, progressive motility and HDS showed a gradual decrease, while sperm concentration and DFI showed a gradual increase. The total sperm count declined starting from 31–35 years.

To explore the possible relationships between semen parameters and DFI or HDS, Pearson's correlation was performed (TABLE 2). DFI was negatively correlated with sperm concentration, total sperm count, total motility, progressive motility and normal morphology ($r = -0.126, -0.072, -0.382, -0.397, -0.203$, respectively, $P < 0.001$) and was positively correlated with semen volume and HDS ($r = 0.084$ and 0.124, respectively, $P < 0.001$). HDS was negatively correlated with all other

TABLE 1 DESCRIPTIVE STATISTICS OF SEMEN PARAMETERS, DFI AND HDS BY MALE AGE CATEGORY

Parameters	Total	≤25 years	26–30 years	31–35 years	36–40 years	41–45 years	46–50 years	>50 years	*P*-value
n	**18,441**	**483**	**4818**	**6920**	**3946**	**1509**	**549**	**216**	
Volume (ml)	2.8	3.0	3.0	3.0	2.6	2.4	2.2	2.0	<0.001
Median (Q25, Q75)	2.0, 3.8	2.0, 4.0	2.0, 4.0	2.0, 4.0	2.0, 3.6	1.8, 3.4	1.4, 3.0	1.2, 3.0	
Mean	2.97	3.16	3.13	3.06	2.86	2.64	2.40	2.15	
SD	1.41	1.42	1.42	1.39	1.41	1.39	1.32	1.35	
Concentration (10^6/ml)	56.52	49.17	55.04	56.52	57.26	59.98	64.42	70.92	<0.001
Median (Q25, Q75)	30.61, 94.12	26.53, 80.17	29.90, 91.09	30.89, 92.80	30.46, 96.69	32.46, 100.21	32.02, 110.08	29.18, 126.25	
Mean	70.98	59.74	68.52	69.91	72.57	76.13	83.40	89.02	
SD	58.55	47.44	55.82	56.51	60.83	63.00	73.51	74.31	
Total sperm count	149.9	136.3	156.2	156.0	141.1	139.2	135.6	118.3	<0.001
Median (Q25, Q75)	76.7, 262.9	71.8, 233.5	79.5, 269.0	82.0, 265.6	72.2, 260.6	70.8, 247.8	60.3, 243.9	52.4, 216.2	
Mean	195.57	176.00	202.24	198.66	191.41	185.34	183.16	170.54	
SD	174.17	151.19	181.49	170.71	174.83	170.53	171.46	175.00	
Motility (%)	25.10	28.58	27.15	25.64	23.98	21.21	20.43	14.05	<0.001
Median (Q25, Q75)	14.22, 39.22	18.33, 43.90	16.05, 41.83	14.68, 39.49	13.13, 38.02	12.11, 35.13	10.58, 32.40	6.79, 24.85	
Mean	27.99	31.78	29.88	28.35	26.86	25.14	23.27	18.87	
SD	17.63	18.67	18.01	17.45	17.30	16.92	16.44	17.10	
Progressive motility (%)	22.37	25.54	24.52	22.82	21.29	18.76	17.16	11.97	<0.001
Median (Q25, Q75)	12.20, 35.68	15.13, 40.85	13.92, 38.28	12.67, 36.01	11.21, 34.37	10.29, 31.79	8.62, 28.47	5.39, 22.07	
Mean	25.27	28.98	27.12	25.58	24.17	22.57	20.69	16.55	
SD	16.65	18.04	17.14	16.47	16.20	15.93	15.16	15.44	
Morphology (%)	2.86	2.75	2.84	2.87	2.86	2.87	2.91	2.87	0.012
Median (Q25, Q75)	1.91, 4.17	1.62, 3.76	1.90, 4.07	1.92, 4.19	1.92, 4.15	1.90, 4.19	1.95, 4.29	1.93, 4.23	
Mean	3.18	2.88	3.14	3.21	3.18	3.20	3.30	3.17	
SD	1.92	1.74	1.92	1.92	1.94	1.93	1.84	1.85	
DFI (%)	15.30	11.33	13.15	14.83	16.60	19.54	22.52	28.77	<0.001
Median (Q25, Q75)	9.25, 24.62	6.81, 18.85	8.02, 21.09	9.06, 23.63	9.99, 25.95	12.38, 31.55	13.88, 34.40	18.35, 43.17	
Mean	18.68	14.60	16.27	17.98	19.80	23.54	25.78	31.70	
SD	12.88	10.91	11.36	12.28	12.99	14.81	15.65	16.83	
HDS (%)	7.44	8.34	7.66	7.46	7.20	7.30	6.73	6.34	<0.001
Median (Q25, Q75)	5.12, 10.62	5.51, 12.14	5.35, 11.00	5.17, 10.76	4.96, 10.18	4.99, 9.97	4.65, 9.78	3.78, 8.78	
Mean	8.51	9.45	8.87	8.57	8.18	8.15	7.76	6.88	
SD	4.98	5.31	5.20	5.04	4.68	4.69	4.57	4.08	

Data are presented as the median (Q25, Q75) and mean ± SD.

Kruskal–Wallis single-factor ANOVA was used to compare the median values of groups.

DFI = DNA fragmentation index; HDS = high DNA stainability.

parameters in this study except semen volume (r = –0.013, P = 0.074) and DFI (r = 0.124, P < 0.001). Total motility and progressive motility had stronger correlations with DFI than with HDS (–0.382 versus –0.091, –0.379 versus –0.086, respectively, P < 0.001), while sperm concentration and total sperm count had stronger correlations with HDS than DFI (–0.218 versus –0.126, –0.212 versus –0.072, respectively, P < 0.001).

TABLE 3 shows that the older group (≥40 years), either in the entire cohort, in the subset with abnormal semen parameters or in the subset with normozoospermia, had a significantly higher frequency of sperm DFI >15% than the younger group (<40 years) (66.65% versus 48.24%, 70.17% versus 53.54%, and 42.29% versus 27.34%, respectively). Likewise, the ≥40 years group had a higher sperm DFI than the <40 years group in the three categories (OR 2.145, 2.042, 1.948, respectively, P < 0.001) (TABLE 3). The ≥40 years group in the entire cohort and in the subset with abnormal semen parameters had a significantly lower prevalence of sperm HDS >15% than the <40 years group (7.39% versus 10.00% and 7.71% versus 10.99%, respectively). The ≥40 years group had a lower HDS than the <40 years group in both the entire cohort and the subset with abnormal semen parameters (OR 0.719 and 0.677, respectively, P < 0.001).

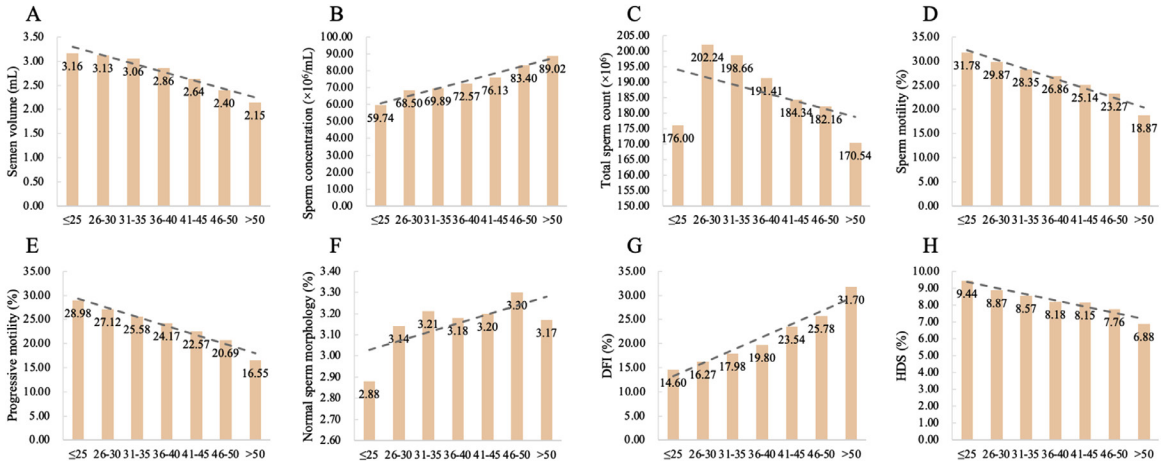

FIGURE 1 Trends in the average semen parameters and their correlations with age. (A) Correlation of semen volume with male age (r = –0.138, P < 0.001). (B) Correlation of sperm concentration with male age (r = 0.065, P < 0.001). (C) Correlation of total sperm count with male age (r = –0.030, P < 0.001). (D) Correlation of sperm motility with male age (r = –0.119, P < 0.001). (E) Correlation of progressive motility with male age (r = –0.121, P < 0.001). (F) Correlation of the normal sperm morphology rate with male age (r = 0.020, P = 0.012). (G) Correlation of sperm DFI (%) with male age (r = 0.225, P < 0.001). (H) Correlation of HDS (%) with male age (r = –0.076, P < 0.001). DFI = DNA fragmentation index; HDS = high DNA stainability.

However, the prevalence of sperm HDS was not significantly different in the subset with normal semen parameters.

DISCUSSION

This is thought to be one of the largest studies ever reported to analyse the effects of ageing on semen traditional parameters, DFI and HDS at the same time. Moreover, the sperm analyses were performed by CASA (computer-aided sperm analysis) and SCSA in the same lab, reducing manipulation differences between technologists. The SCSA test was first described in 1980 (*Evenson et al., 1980*) and then validated with numerous animal fertility studies and various toxicology and biochemical studies (*Evenson, 2016*). SCSA data show high correlations with in-vivo human fertility (*Evenson et al., 1999*) and IUI (*Bungum et al., 2004*) and IVF/ICSI (*Oleszczuk et al., 2016*) pregnancy outcomes.

Ageing is known to be one of the highest risk factors for human diseases, including cancer, diabetes, neurodegeneration and metabolic syndrome. In the field of ART, the association between advanced maternal age and adverse reproductive outcomes, such as declines in fertility, increased risks of spontaneous abortion, pregnancy complications, congenital anomalies and perinatal mortality, is well established (*Belloc et al., 2008*; *Heffner, 2004*; *Maheshwari et al., 2008*). Comparatively, evidence showing that male age has an impact on the outcomes of ART and offspring fitness is increasing. Ageing may impair the quantity and quality of spermatozoa, leading to potential abnormalities in fertilization, embryo development, implantation and pregnancy outcomes.

TABLE 2 CORRELATION OF TRADITIONAL SEMEN PARAMETERS WITH DFI AND HDS

Variables	DFI		HDS	
	r	*P*-value	*r*	*P*-value
Semen volume	0.084	<0.001	–0.013	0.074
Sperm concentration	–0.126	<0.001	–0.218	<0.001
Total sperm count	–0.072	<0.001	–0.212	<0.001
Total motility	–0.382	<0.001	–0.091	<0.001
Progressive motility	–0.379	<0.001	–0.086	<0.001
Normal morphology	–0.203	<0.001	–0.283	<0.001
HDS	0.124	<0.001	–	–

DFI = DNA fragmentation index; HDS = high DNA stainability.

Conventional semen characteristics such as semen volume, sperm concentration, total sperm count, morphology, total motility and progressive motility are commonly measured to evaluate semen quality and quantity. Numerous studies have reported that advancing paternal age has a negative impact on semen volume (*Sloter et al., 2006*; *Winkle et al., 2009*), sperm motility (*Sloter et al., 2006*) and total sperm count (*Paoli et al., 2019*; *Sloter et al., 2006*; *Stone et al., 2013*; *Veron et al., 2018*). However, there is no consistent evidence regarding the effect of ageing on sperm concentration and normal sperm morphology.

In previous studies, semen parameters were either studied by a single selected age threshold (e.g. age 40 years) (*Alshahrani et al., 2014*; *Kaarouch et al., 2018*; *Rosiak-Gill et al., 2019*) or compared between older patients and chronologically remote young patients. Few studies have assessed semen parameters from a continuous age spectrum (*Eskenazi et al., 2003*; *Wyrobek et al., 2006*), and their sample sizes were small. Therefore, this study obtained a sufficiently large sample size and measured and compared semen parameters across a continuous age spectrum to better understand the effects of ageing on male fertility.

In the current study, the peaks and trends of conventional semen parameters with age were largely consistent with those of previous studies (*Begueria*

TABLE 3 PREVALENCE OF DFI AND HDS IN THE GROUP OF MALES AGED ≥40 YEARS AND <40 YEARS

Group	<40 years *n* (%)	≥40 years *n* (%)	OR (95% CI)
The entire cohort			
DFI ≤15%	8109 (51.76)	925 (33.35)[a]	0.466[a] (0.428–0.508)
DFI >15%	7558 (48.24)	1849 (66.65)[a]	2.145[a] (1.970–2.335)
HDS ≤15%	14,101 (90.00)	2569 (92.61)[a]	1.353[a] (1.176–1.556)
HDS >15%	1566 (10.00)	205 (7.39)[a]	0.719[a] (0.618–0.836)
Abnormal semen parameters			
DFI ≤15%	5807 (46.46)	723 (29.83)[a]	0.490[a] (0.446–0.538)
DFI >15%	6692 (53.54)	1701 (70.17)[a]	2.042[a] (1.859–2.242
HDS ≤15%	11,125 (89.01)	2237 (92.29)[a]	1.398[a] (1.215–1.607)
HDS >15%	1374 (10.99)	187 (7.71)[a]	0.677[a] (0.577–0.794)
Normal semen parameters			
DFI ≤15%	2302 (72.66)	202 (57.71)[a]	0.513[a] (0.410–0.644)
DFI >15%	866 (27.34)	148 (42.29)[a]	1.948[a] (1.554–2.441)
HDS ≤15%	2976 (93.94)	332 (94.86)	1.178 (0.736–1.886)
HDS >15%	192 (6.06)	18 (5.14)	0.840 (0.512–1.380)

Normal semen parameters were considered according to the following criteria: semen volume ≥1.5 ml, sperm concentration ≥15 × 10^6/ml, total sperm count ≥39 × 10^6, sperm progressive motility ≥32%, total motility ≥40%. If one of the criteria is not met, it is considered an abnormal semen parameter.

CI = confidence interval; DFI = DNA fragmentation index; HDS = high DNA stainability; OR = odds ratio.

[a] Significant difference between compared groups at $P < 0.001$; chi-squared test.

et al., 2014; *Levitas et al., 2007*; *Salmon-Divon et al., 2020*). Advanced age had a negative impact on semen volume, total sperm count, total motility and progressive motility and a positive impact on sperm concentration. Regarding normal sperm morphology, previous studies have made inconsistent observations. While some reported that normal morphology had no relationship with age (*Begueria et al., 2014*; *Brahem et al., 2011*; *Das et al., 2013*; *Hossain et al., 2012*; *Veron et al., 2018*), others suggested that men aged >50 years had fewer spermatozoa with normal morphology than those aged 20–32 years (*Paoli et al., 2019*). However, in this study, although the correlation of the normal sperm morphology rate with male age was significant ($r = 0.020$, $P = 0.012$), comparison between groups showed that only the percentage of normal morphology in the 46–50 years group was higher than that in the ≤25 years group ($P = 0.014$), while no significant differences were found among the other groups (Supplementary Table 1).

Ageing induces profound alterations in the male reproductive system, namely in the hypothalamus pituitary testicular (HPT) axis, which consequently compromises testicular morphology and physiology. The HPT axis controls the release of sex hormones and ensures the initiation and progression of spermatogenesis. Indeed, both the hormonal profile of sex steroids and spermatogenesis suffer gradual changes induced by age-related alterations (*Gunes et al., 2016*). The number of synaptic inputs to gonadotrophin-releasing hormone (GnRH) neurons, as well as both GnRH transcripts and peptides, seems to decrease with age (*Witkin, 1987*). Pituitary secretion of FSH is altered in older men, and an increase in circulating concentrations of FSH has been shown in longitudinal studies with ageing men (*Harman et al., 2001*; *Lapauw et al., 2008*; *Morley et al., 1997*). Age was a negative determinant of LH secretory burst amplitude and a positive predictor of LH secretory burst frequency and basal LH secretory rates (*Veldhuis et al., 1992*), while others suggested that the pattern of LH release was completely chaotic in older men (*Pincus et al., 1996*). A possible explanation is that ageing is associated with impaired pituitary responsiveness to GnRH. Then, the testicular production of testosterone will certainly be influenced by these disturbances in the hypothalamic–pituitary complex. Testosterone concentrations decrease with advancing age (*Harman et al., 2001*), due not only to disturbances in the circuits of the HPT axis but also to impairment in the number of Leydig cells and deterioration of testicular function. Leydig cells are responsible for the production of testosterone in the testis. Ageing in the human testes is associated with a reduction in the numbers and functional competence of Leydig cells (*Cummins et al., 1994*). Several age-associated changes have been reported in the hormonal profile and testicular physiology, including alterations in the testes (seminiferous tubular narrowing, vacuolization of Sertoli cells, decreased Leydig cell number and testosterone production), mitochondrial dysfunction, atherosclerotic alterations in testicular arteries, decreased testicular volume and germ cell depletion (*Almeida et al., 2017*; *Centola and Eberly, 1999*). Due to these hormonal patterns, testicular physiology and cell function alterations, older men exhibited impaired spermatogenesis, the total sperm count appeared to decline, and percentage motility decreased.

The current study showed that while the total sperm count and semen volume declined with age, the sperm concentration increased. In fact, sperm concentration is the number of spermatozoa per millilitre, which depends on both the total sperm count and semen volume. If the semen volume decreases faster than the total sperm count decreases, the resulting sperm concentration would be higher. The

absolute value of Pearson's correlation coefficient between age and semen volume was calculated here, which was higher than that between age and total sperm count (0.138 versus 0.030). Therefore, it is suggested that the increasing sperm concentration with age might result from the faster rate of semen volume decrease. A semen volume decrease could be caused by seminal vesicle insufficiency or prostate disease, which often occur in older men (*Rui et al., 1986*; *Thurmond et al., 2015*).

There is growing concern about how the diagnosis of sperm DNA integrity should be used in the clinical courses of infertile couples. Sperm DNA fragmentation and uncondensed chromatin, measured with the DFI and HDS, are increasingly recognized as important predictors of DNA damage and clinical outcomes. *Deenadayal Mettler et al. (2019)* reported that while DFI had a weak positive correlation with age, HDS showed a weak negative correlation. Comparatively, this study showed the same correlation: DFI was positively correlated with age (r = 0.225, P < 0.001), and HDS was negatively correlated with age (r = –0.076, P < 0.001). These correlations were weak but significant. In addition, this study showed that HDS was positively correlated with DFI (r = 0.124, P < 0.001), consistent with the study by *Richthoff et al. (2002)* (r = 0.18, P = 0.002). However, it should be noted that sperm DNA fragmentation and nuclear decondensation are independent processes, and each value must be considered independently in treatment management.

Furthermore, this study investigated the correlations between sperm DNA fragmentation and conventional sperm parameters. Previous studies have shown inconsistent evidence regarding their relationships. *Yuan et al. (2019)* showed that sperm viability, progressive motility and normal morphology were negatively correlated with DFI and that sperm counts had no significant relationship with DFI. *Evgeni et al. (2015)* reported a significant inverse correlation between DNA fragmentation and sperm concentration, total count, progressive motility and normal morphology (P < 0.05). *Xie et al. (2018)* found that the sperm DFI had no correlation with conventional sperm parameters. In this study, DFI was inversely correlated with sperm concentration, total sperm count, total motility, progressive motility and normal morphology. Comparatively, DFI had positive associations with age, semen volume and HDS (P < 0.001). The results were consistent with some earlier studies (*Le et al., 2019*; *Smit et al., 2010*). In addition, HDS had inverse correlations with sperm concentration, total sperm count, total motility, progressive motility and normal morphology (P < 0.001), but not semen volume (r = –0.013, P = 0.074).

The mechanisms of age-dependent patterns of decline in sperm fitness are not fully understood. Protamination failures, apoptosis and oxidative stress are three possible causes of chromatin damage and sperm DNA fragmentation. Oxidative stress occurs when there is an imbalance between reactive oxygen species (ROS) production and antioxidants in seminal plasma and is thought to be an important contributor (*Aitken and De Iuliis, 2010*). ROS play an important role in sperm maturation and capacitation (*Aitken and Curry, 2011*), which are critical for successful fertilization under normal physiological conditions (*Aitken, 2011*; *Drevet and Aitken, 2019*). However, excessive production of ROS is related to abnormal semen parameters and sperm damage (*Deepinder et al., 2008*; *Muratori et al., 2019*) and is associated with impaired sperm fertilizing ability and lower pregnancy rates after IVF (*Zorn et al., 2003*). More studies have shown that sperm DNA damage is associated with high ROS (*Nguyen-Powanda and Robaire, 2020*; *Vaughan et al., 2020*). One specific mechanism of oxidative processes is to condense the sperm nucleus by creating inter- and intramolecular cross bridges between and within nuclear protamines and ultimately locking it into a compacted state, completed in the epididymis (*Drevet, 2006*; *Dutta et al., 2019*). Therefore, excessive production of ROS may transiently increase the nuclear condensation of sperm cells and simultaneously promote spontaneous DNA fragmentation (*Drevet and Aitken, 2019*). In the process of ageing, oxidative stress increases by the overaccumulation of ROS (*Gunes et al., 2016*). In healthy fertile men older than 40 years, ROS concentrations are significantly higher in the seminal ejaculates (*Cocuzza et al., 2008*). HDS was negatively correlated with age, and DFI was positively correlated with age, which may be explained by ROS-induced DNA compaction (low HDS) and abnormal DNA fragmentation (high DFI). *Vaughan et al. (2020)* found that sperm DFI and oxidative status increased with age, and HDS decreased with age in a large cohort of 16,945 males. There was a significant linear trend for DFI, OSA and HDS across the age categories. This is consistent with the current findings and provides more understanding of sperm DNA damage. The DNA damage in spermatozoa may originate from spermiogenesis, while another population of DNA-damaged spermatozoa is processed correctly during spermatogenesis but is disrupted by ROS during transport in the reproductive tract, which may be the primary cause of sperm DNA damage in older men, because the ROS accumulates with ageing. However, *Vaughan et al. (2020)* found that the percentage of high oxidative stress adducts patients in the high DFI group decreased by age, suggesting that defective spermatogenesis may be more prevalent in older men. Nevertheless, the mechanism of the complex relationship between ROS and sperm DNA damage is still unclear. In addition, the number of spermatozoa with chromatin immaturity was significantly higher in the short ejaculatory abstinence group than in the long ejaculatory abstinence group (*Uppangala et al., 2016*), and the percentage of sperm protamination was significantly increased with abstinence (*Comar et al., 2017*). It was reported that sperm DFI is higher in longer ejaculatory abstinence (*Comar et al., 2017*; *Gosalvez et al., 2011*). Considering this in this way, the higher DFI and lower HDS in older patients are probably because the abstinence time in older age groups may be longer than that in the young group. An increase in the number of dysfunctional spermatozoa in semen significantly induces higher ROS production, affecting mitochondrial function and motility. In animal studies, the abnormally high concentration of ROS resulted in oxidative damage to mitochondrial function and accelerated mitochondria-dependent apoptosis, which could be the main reason for the decline in sperm motility (*Liu et al., 2019*; *Slowinska et al., 2018*). Therefore, from the current data and previous studies, it is suggested that ageing may cause accumulative oxidative stress that impairs the fertilization potential of spermatozoa,

damages DNA, and even affects the epigenetic profile of sperm cells (*Aitken, 2016*; *Aitken et al., 2014*).

Furthermore, the current study demonstrated that most semen parameters began to change as early as 30–35 years and that the change became more rapid after 40 years. Similarly, DFI increased with age, especially above the age of 40. Multiple studies demonstrating that sperm DFI increased with advancing male age used 40 years as a threshold (*Das et al., 2013*; *Rosiak-Gill et al., 2019*; *Veron et al., 2018*). They reported a higher incidence of sperm DNA damage (>10% DFI) in individuals aged ≥40 years than in those aged <40 years. Older men had more than twice the odds of high sperm DNA damage as younger men (*Rosiak-Gill et al., 2019*). IVF outcomes were also affected by paternal age, indicated by the rates of cancelled embryo transfers, clinical pregnancy and miscarriage in the advanced age group compared with the young group (29%, 17% and 60% versus 10%, 32% and 42%). Therefore, the age of 40 years was suggested to be the advanced paternal age cut-off for ART attempts (*Kaarouch et al., 2018*). A study by *Das et al. (2013)* evaluated the relationship between sperm DNA damage, conventional semen parameters and paternal age in 277 consecutive non-azoospermic men and compared the prevalence of isolated sperm DNA damage in <40-year-old and ≥40-year-old men. They found that sperm DFI was positively correlated with paternal age and inversely correlated with progressive motility. Sperm DFI was significantly higher in normozoospermic men who were aged ≥40 years than in those aged <40 years, and the prevalence of >30% DFI was significantly higher in older normozoospermic men than in younger normozoospermic men. The current findings regarding DFI prevalence are consistent with those of previous studies. Moreover, this study showed that males of advanced paternal age (≥40 years) had decreased sperm HDS in the entire cohort and in the subset with abnormal semen parameters (7.39% versus 10.00% and 7.71% versus 10.99%, respectively; OR 0.719 and 0.677, respectively; $P < 0.001$). However, the prevalence of sperm HDS was not significantly different between the two age groups with normal semen parameters.

The current findings indicate weak but significant correlations of advanced age with sperm conventional characteristics and sperm DNA/chromatin integrity, suggesting that fertility may decline with increasing age. However, individual variabilities, testicular structure and function, spermatogenesis, transport in the reproductive tract, tolerance of sperm to external negative factors after ejaculation, and many other factors make it difficult to conclusively determine the relationship between age and semen parameters. Older men may have worse sperm quality and quantity, lower fecundity, and higher risks of gene-associated diseases in their offspring. However, factors such as female age, lifestyle and diseases should also be taken into account to predict the outcomes of reproduction.

This study has some limitations, including its retrospective nature, the possibility of sampling error in the measurements, and the inability to obtain height, weight, lifestyle and therapy status information from the subjects. The data in this study were collected in the Andrology Laboratory of the Reproductive Medicine Center, which may not represent the trend for changes in sperm quality in the overall male population. The lack of a fertile population for comparison in each age group and the lack of ART outcome data were also weaknesses of the study. Nevertheless, the data provide useful information for those interested in studying changes in semen with ageing and offer a basic reference for clinical decisions.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.rbmo.2021.02.006.

REFERENCES

Aboulghar, M., Mansour, R., Al-Inany, H., Abou-Setta, A.M., Aboulghar, M., Mourad, L., Serour, G. **Paternal age and outcome of intracytoplasmic sperm injection.** Reprod. Biomed. Online 2007; 14: 588–592

Aitken, R.J. **The capacitation-apoptosis highway: oxysterols and mammalian sperm function.** Biol. Reprod. 2011; 85: 9–12

Aitken, R.J. **Oxidative stress and the etiology of male infertility.** J. Assist. Reprod. Genet. 2016; 33: 1691–1692

Aitken, R.J., Curry, B.J. **Redox regulation of human sperm function: from the physiological control of sperm capacitation to the etiology of infertility and DNA damage in the germ line.** Antioxid. Redox Signal 2011; 14: 367–381

Aitken, R.J., De Iuliis, G.N. **On the possible origins of DNA damage in human spermatozoa.** Mol. Hum. Reprod. 2010; 16: 3–13

Aitken, R.J., Smith, T.B., Jobling, M.S., Baker, M.A., De Iuliis, G.N. **Oxidative stress and male reproductive health.** Asian. J. Androl. 2014; 16: 31–38

Almeida, S., Rato, L., Sousa, M., Alves, M.G., Oliveira, P.F. **Fertility and sperm quality in the aging male.** Curr. Pharm. Des. 2017; 23: 4429–4437

Alshahrani, S., Agarwal, A., Assidi, M., Abuzenadah, A.M., Durairajanayagam, D., Ayaz, A., Sharma, R., Sabanegh, E. **Infertile men older than 40 years are at higher risk of sperm DNA damage.** Reprod. Biol. Endocrinol. 2014; 12: 103

Begueria, R., Garcia, D., Obradors, A., Poisot, F., Vassena, R., Vernaeve, V. **Paternal age and assisted reproductive outcomes in ICSI donor oocytes: is there an effect of older fathers?** Hum. Reprod. 2014; 29: 2114–2122

Belloc, S., Cohen-Bacrie, P., Benkhalifa, M., Cohen-Bacrie, M., De Mouzon, J., Hazout, A., Menezo, Y. **Effect of maternal and paternal age on pregnancy and miscarriage rates after intrauterine insemination.** Reprod. Biomed. Online 2008; 17: 392–397

Bellver, J., Garrido, N., Remohi, J., Pellicer, A., Meseguer, M. **Influence of paternal age on assisted reproduction outcome.** Reprod. Biomed. Online 2008; 17: 595–604

Brahem, S., Mehdi, M., Elghezal, H., Saad, A. **The effects of male aging on semen quality, sperm DNA fragmentation and chromosomal abnormalities in an infertile population.** J. Assist. Reprod. Genet. 2011; 28: 425–432

Bungum, M., Humaidan, P., Spano, M., Jepson, K., Bungum, L., Giwercman, A. **The predictive value of sperm chromatin structure assay (SCSA) parameters for the outcome of intrauterine insemination, IVF and ICSI.** Hum. Reprod. 2004; 19: 1401–1408

Centola, G.M., Eberly, S. **Seasonal variations and age-related changes in human sperm count, motility, motion parameters, morphology, and white blood cell concentration.** Fertil. Steril. 1999; 72: 803–808

Cocuzza, M., Athayde, K.S., Agarwal, A., Sharma, R., Pagani, R., Lucon, A.M., Srougi, M., Hallak, J. **Age-related increase of reactive oxygen species in neat semen in healthy fertile men.** Urology 2008; 71: 490–494

Comar, V.A., Petersen, C.G., Mauri, A.L., Mattila, M., Vagnini, L.D., Renzi, A., Petersen, B., Nicoletti, A., Dieamant, F., Oliveira, J.B.A.,

Baruffi, R.L.R., Franco, J.G.Jr. **Influence of the abstinence period on human sperm quality: analysis of 2,458 semen samples.** JBRA Assist. Reprod. 2017; 21: 306–312

Conti, S.L., Eisenberg, M.L. **Paternal aging and increased risk of congenital disease, psychiatric disorders, and cancer.** Asian J. Androl. 2016; 18: 420–424

Cummins, J.M., Jequier, A.M., Kan, R. **Molecular biology of human male infertility: links with aging, mitochondrial genetics, and oxidative stress?** Mol. Reprod. Dev. 1994; 37: 345–362

Das, M., Al-Hathal, N., San-Gabriel, M., Phillips, S., Kadoch, I.J., Bissonnette, F., Holzer, H., Zini, A. **High prevalence of isolated sperm DNA damage in infertile men with advanced paternal age.** J. Assist. Reprod. Genet. 2013; 30: 843–848

de La Rochebrochard, E., de Mouzon, J., Thepot, F., Thonneau, P. **French National IVF Registry (FIVNAT) Association. Fathers over 40 and increased failure to conceive: the lessons of in vitro fertilization in France.** Fertil. Steril. 2006; 85: 1420–1424

Deenadayal Mettler, A., Govindarajan, M., Srinivas, S., Mithraprabhu, S., Evenson, D., Mahendran, T. **Male age is associated with sperm DNA/chromatin integrity.** Aging Male 2019: 1–8

Deepinder, F., Cocuzza, M., Agarwal, A. **Should seminal oxidative stress measurement be offered routinely to men presenting for infertility evaluation?** Endocr. Pract. 2008; 14: 484–491

Drevet, J.R. **The antioxidant glutathione peroxidase family and spermatozoa: a complex story.** Mol. Cell. Endocrinol. 2006; 250: 70–79

Drevet, J.R., Aitken, R.J. **Oxidative damage to sperm DNA: attack and defense.** Adv. Exp. Med. Biol. 2019; 1166: 107–117

Dutta, S., Majzoub, A., Agarwal, A. **Oxidative stress and sperm function: A systematic review on evaluation and management.** Arab. J. Urol. 2019; 17: 87–97

Eskenazi, B., Wyrobek, A.J., Sloter, E., Kidd, S.A., Moore, L., Young, S., Moore, D **The association of age and semen quality in healthy men.** Hum. Reprod. 2003; 18: 447–454

Evenson, D.P. **The Sperm Chromatin Structure Assay (SCSA(®)) and other sperm DNA fragmentation tests for evaluation of sperm nuclear DNA integrity as related to fertility.** Anim. Reprod. Sci. 2016; 169: 56–75

Evenson, D.P. **Evaluation of sperm chromatin structure and DNA strand breaks is an important part of clinical male fertility assessment.** Transl. Androl. Urol. 2017; 6: S495–S500

Evenson, D. P. 2018 Sperm chromatin structure assay (SCSA®): Evolution from origin to clinical utility. In: Zini, A. and Agarwal, A. editors. Springer International

Evenson, D., Darzynkiewicz, Z., Jost, L., Janca, F., Ballachey, B. **Changes in accessibility of DNA to various fluorochromes during spermatogenesis.** Cytometry 1986; 7: 45–53

Evenson, D.P., Darzynkiewicz, Z., Melamed, M.R. **Relation of mammalian sperm chromatin heterogeneity to fertility.** Science 1980; 210: 1131–1133

Evenson, D.P., Jost, L.K., Baer, R.K., Turner, T.W., Schrader, S.M. **Individuality of DNA denaturation patterns in human sperm as measured by the sperm chromatin structure assay.** Reprod. Toxicol. 1991; 5: 115–125

Evenson, D.P., Jost, L.K., Marshall, D., Zinaman, M.J., Clegg, E., Purvis, K., de Angelis, P., Claussen, O.P. **Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic.** Hum. Reprod. 1999; 14: 1039–1049

Evenson, D.P., Djira, G., Kasperson, K., Christianson, J. **Relationships between the age of 25,445 men attending infertility clinics and sperm chromatin structure assay (SCSA(R)) defined sperm DNA and chromatin integrity.** Fertil. Steril. 2020; 114: 311–320

Evgeni, E., Lymberopoulos, G., Gazouli, M., Asimakopoulos, B. **Conventional semen parameters and DNA fragmentation in relation to fertility status in a Greek population.** Eur. J. Obstet. Gynecol. Reprod. Biol. 2015; 188: 17–23

Ferreira, R.C., Braga, D.P., Bonetti, T.C., Pasqualotto, F.F., Iaconelli, A.Jr, Borges, E.Jr **Negative influence of paternal age on clinical intracytoplasmic sperm injection cycle outcomes in oligozoospermic patients.** Fertil. Steril. 2010; 93: 1870–1874

Fragouli, E., Wells, D., Thornhill, A., Serhal, P., Faed, M.J., Harper, J.C., Delhanty, J.D. **Comparative genomic hybridization analysis of human oocytes and polar bodies.** Hum. Reprod. 2006; 21: 2319–2328

Franasiak, J.M., Forman, E.J., Hong, K.H., Werner, M.D., Upham, K.M., Treff, N.R., Scott, R.T.Jr. **The nature of aneuploidy with increasing age of the female partner: a review of 15,169 consecutive trophectoderm biopsies evaluated with comprehensive chromosomal screening.** Fertil. Steril. 2014; 101

Girsh, E., Katz, N., Genkin, L., Girtler, O., Bocker, J., Bezdin, S., Barr, I. **Male age influences oocyte-donor program results.** J. Assist. Reprod. Genet. 2008; 25: 137–143

Gosalvez, J., Gonzalez-Martinez, M., Lopez-Fernandez, C., Fernandez, J.L., Sanchez-Martin, P. **Shorter abstinence decreases sperm deoxyribonucleic acid fragmentation in ejaculate.** Fertil. Steril. 2011; 96: 1083–1086

Gunes, S., Hekim, G.N., Arslan, M.A., Asci, R. **Effects of aging on the male reproductive system.** J. Assist. Reprod. Genet. 2016; 33: 441–454

Guo, L.Y., Zhou, H., Liu, M., Li, Q., Sun, X.F. **Male age is more critical to sperm DNA integrity than routine semen parameters in Chinese infertile males.** Andrologia 2020; 52: e13449

Harman, S.M., Metter, E.J., Tobin, J.D., Pearson, J., Blackman, M.R. **and Baltimore Longitudinal Study of Aging. Longitudinal effects of aging on serum total and free testosterone levels in healthy men. Baltimore Longitudinal Study of Aging.** J. Clin. Endocrinol. Metab. 2001; 86: 724–731

Heffner, L.J. **Advanced maternal age–how old is too old?** N Engl J Med 2004; 351: 1927–1929

Hellstrom, W.J., Overstreet, J.W., Sikka, S.C., Denne, J., Ahuja, S., Hoover, A.M., Sides, G.D., Cordell, W.H., Harrison, L.M., Whitaker, J.S. **Semen and sperm reference ranges for men 45 years of age and older.** J. Androl. 2006; 27: 421–428

Horta, F., Vollenhoven, B., Healey, M., Busija, L., Catt, S., Temple-Smith, P. **Male ageing is negatively associated with the chance of live birth in IVF/ICSI cycles for idiopathic infertility.** Hum. Reprod. 2019; 34: 2523–2532

Hossain, M.M., Fatima, P., Rahman, D., Hossain, H.B. **Semen parameters at different age groups of male partners of infertile couples.** Mymensingh Med. J. 2012; 21: 306–315

Humm, K.C., Sakkas, D. **Role of increased male age in IVF and egg donation: is sperm DNA fragmentation responsible?** Fertil. Steril. 2013; 99: 30–36

Kaarouch, I., Bouamoud, N., Madkour, A., Louanjli, N., Saadani, B., Assou, S., Aboulmaouahib, S., Amzazi, S., Copin, H., Benkhalifa, M., Sefrioui, O. **Paternal age: Negative impact on sperm genome decays and IVF outcomes after 40 years.** Mol. Reprod. Dev. 2018; 85: 271–280

Khandwala, Y.S., Zhang, C.A., Lu, Y., Eisenberg, M.L. **The age of fathers in the USA is rising: an analysis of 168 867 480 births from 1972 to 2015.** Hum. Reprod. 2017; 32: 2110–2116

Khandwala, Y.S., Baker, V.L., Shaw, G.M., Stevenson, D.K., Lu, Y., Eisenberg, M.L. **Association of paternal age with perinatal outcomes between 2007 and 2016 in the United States: population based cohort study.** BMJ 2018; 363: k4372

Kimura, R., Yoshizaki, K., Osumi, N. **Risk of Neurodevelopmental Disease by Paternal Aging: A Possible Influence of Epigenetic Alteration in Sperm.** Adv. Exp. Med. Biol. 2018; 1012: 75–81

Klonoff-Cohen, H.S., Natarajan, L. **The effect of advancing paternal age on pregnancy and live birth rates in couples undergoing in vitro fertilization or gamete intrafallopian transfer.** Am. J. Obstet. Gynecol. 2004; 191: 507–514

Kuliev, A., Cieslak, J., Ilkevitch, Y., Verlinsky, Y. **Chromosomal abnormalities in a series of 6,733 human oocytes in preimplantation diagnosis for age-related aneuploidies.** Reprod. Biomed. Online 2003; 6: 54–59

Lapauw, B., Goemaere, S., Zmierczak, H., Van Pottelbergh, I., Mahmoud, A., Taes, Y., De Bacquer, D., Vansteelandt, S., Kaufman, J.M. **The decline of serum testosterone levels in community-dwelling men over 70 years of age: descriptive data and predictors of longitudinal changes.** Eur. J. Endocrinol. 2008; 159: 459–468

Le, M.T., Nguyen, T.A.T., Nguyen, H.T.T., Nguyen, T.T.T., Nguyen, V.T., Le, D.D., Nguyen, V.Q.H., Cao, N.T. **Does sperm DNA fragmentation correlate with semen parameters?** Reprod. Med. Biol. 2019; 18: 390–396

Levitas, E., Lunenfeld, E., Weisz, N., Friger, M., Potashnik, G. **Relationship between age and semen parameters in men with normal sperm concentration: analysis of 6022 semen samples.** Andrologia 2007; 39: 45–50

Liu, T., Han, Y., Zhou, T., Zhang, R., Chen, H., Chen, S., Zhao, H. **Mechanisms of ROS-induced mitochondria-dependent apoptosis underlying liquid storage of goat spermatozoa.** Aging (Albany NY) 2019; 11: 7880–7898

Lowe, X., Eskenazi, B., Nelson, D.O., Kidd, S., Alme, A., Wyrobek, A.J. **Frequency of XY sperm increases with age in fathers of boys with Klinefelter syndrome.** Am. J. Hum. Genet. 2001; 69: 1046–1054

Maheshwari, A., Hamilton, M., Bhattacharya, S. **Effect of female age on the diagnostic categories of infertility.** Hum. Reprod. 2008; 23: 538–542

Morley, J.E., Kaiser, F.E., Perry, H.M.3rd, Patrick, P., Morley, P.M., Stauber, P.M., Vellas, B., Baumgartner, R.N., Garry, P.J. **Longitudinal changes in testosterone, luteinizing hormone, and follicle-stimulating hormone in healthy older men.** Metabolism 1997; 46: 410–413

Moskovtsev, S.I., Willis, J., Mullen, J.B. **Age-related decline in sperm deoxyribonucleic acid integrity in patients evaluated for male infertility.** Fertil. Steril. 2006; 85: 496–499

Muratori, M., Marchiani, S., Tamburrino, L., Baldi, E. **Sperm DNA Fragmentation: Mechanisms of Origin.** Adv. Exp. Med. Biol. 2019; 1166: 75–85

Nguyen-Powanda, P., Robaire, B. **Oxidative Stress and Reproductive Function in the Aging Male.** Biology (Basel) 2020; 9: 282

Nijs, M., De Jonge, C., Cox, A., Janssen, M., Bosmans, E., Ombelet, W. **Correlation between male age, WHO sperm parameters, DNA fragmentation, chromatin packaging and outcome in assisted reproduction technology.** Andrologia 2011; 43: 174–179

Oleszczuk, K., Giwercman, A., Bungum, M. **Sperm chromatin structure assay in prediction of in vitro fertilization outcome.** Andrology 2016; 4: 290–296

Paoli, D., Pecora, G., Pallotti, F., Faja, F., Pelloni, M., Lenzi, A., Lombardo, F. **Cytological and molecular aspects of the ageing sperm.** Hum. Reprod. 2019; 34: 218–227

Pincus, S.M., Mulligan, T., Iranmanesh, A., Gheorghiu, S., Godschalk, M., Veldhuis, J.D. **Older males secrete luteinizing hormone and testosterone more irregularly, and jointly more asynchronously, than younger males.** Proc. Natl. Acad. Sci. U. S. A. 1996; 93: 14100–14105

Ramasamy, R., Chiba, K., Butler, P., Lamb, D.J. **Male biological clock: a critical analysis of advanced paternal age.** Fertil. Steril. 2015; 103: 1402–1406

Richthoff, J., Spano, M., Giwercman, Y.L., Frohm, B., Jepson, K., Malm, J., Elzanaty, S., Stridsberg, M., Giwercman, A. **The impact of testicular and accessory sex gland function on sperm chromatin integrity as assessed by the sperm chromatin structure assay (SCSA).** Hum. Reprod. 2002; 17: 3162–3169

Rosiak-Gill, A., Gill, K., Jakubik, J., Fraczek, M., Patorski, L., Gaczarzewicz, D., Kurzawa, R., Kurpisz, M., Piasecka, M. **Age-related changes in human sperm DNA integrity.** Aging (Albany NY) 2019; 11: 5399–5411

Rui, H., Thomassen, Y., Oldereid, N.B., Purvis, K. **Accessory sex gland function in normal young (20–25 years) and middle-aged (50–55 years) men.** J. Androl. 1986; 7: 93–99

Salmon-Divon, M., Shrem, G., Balayla, J., Nehushtan, T., Volodarsky-Perel, A., Steiner, N., Son, W.Y., Dahan, M.H. **An age-based sperm nomogram: the McGill reference guide.** Hum. Reprod. 2020; 35: 2213–2225

Schmidt, L., Sobotka, T., Bentzen, J.G., Nyboe Andersen, A. **ESHRE Reproduction and Society Task Force. Demographic and medical consequences of the postponement of parenthood.** Hum. Reprod. Update 2012; 18: 29–43

Sharma, R., Agarwal, A., Rohra, V.K., Assidi, M., Abu-Elmagd, M., Turki, R.F. **Effects of increased paternal age on sperm quality, reproductive outcome and associated epigenetic risks to offspring.** Reprod. Biol. Endocrinol. 2015; 13: 35

Simon, L., Emery, B., Carrell, D.T. **Sperm DNA Fragmentation: Consequences for Reproduction.** Adv. Exp. Med. Biol. 2019; 1166: 87–105

Sloter, E., Schmid, T.E., Marchetti, F., Eskenazi, B., Nath, J., Wyrobek, A.J. **Quantitative effects of male age on sperm motion.** Hum. Reprod. 2006; 21: 2868–2875

Slowinska, M., Liszewska, E., Judycka, S., Konopka, M., Ciereszko, A. **Mitochondrial membrane potential and reactive oxygen species in liquid stored and cryopreserved turkey (Meleagris gallopavo) spermatozoa.** Poult. Sci. 2018; 97: 3709–3717

Smit, M., Romijn, J.C., Wildhagen, M.F., Weber, R.F., Dohle, G.R. **Sperm chromatin structure is associated with the quality of spermatogenesis in infertile patients.** Fertil. Steril. 2010; 94: 1748–1752

Spandorfer, S.D., Avrech, O.M., Colombero, L.T., Palermo, G.D., Rosenwaks, Z. **Effect of parental age on fertilization and pregnancy characteristics in couples treated by intracytoplasmic sperm injection.** Hum. Reprod. 1998; 13: 334–338

Stone, B.A., Alex, A., Werlin, L.B., Marrs, R.P. **Age thresholds for changes in semen parameters in men.** Fertil. Steril. 2013; 100: 952–958

Thurmond, P., Yang, J.H., Li, Y., Lerner, L.B., Azadzoi, K.M. **Structural modifications of the prostate in hypoxia, oxidative stress, and chronic ischemia.** Korean J. Urol. 2015; 56: 187–196

Uppangala, S., Mathai, S.E., Salian, S.R., Kumar, D., Singh, V.J., D'Souza, F., Kalthur, G., Kamath, A., Adiga, S.K. **Sperm Chromatin Immaturity Observed in Short Abstinence Ejaculates Affects DNA Integrity and Longevity In Vitro.** PLoS One 2016; 11e0152942

Vagnini, L., Baruffi, R.L., Mauri, A.L., Petersen, C.G., Massaro, F.C., Pontes, A., Oliveira, J.B., Franco, J.G.Jr. **The effects of male age on sperm DNA damage in an infertile population.** Reprod. Biomed. Online 2007; 15: 514–519

Varshini, J., Srinag, B.S., Kalthur, G., Krishnamurthy, H., Kumar, P., Rao, S.B., Adiga, S.K. **Poor sperm quality and advancing age are associated with increased sperm DNA damage in infertile men.** Andrologia 2012; 44: 642–649

Vaughan, D.A., Tirado, E., Garcia, D., Datta, V., Sakkas, D. **DNA fragmentation of sperm: a radical examination of the contribution of oxidative stress and age in 16 945 semen samples.** Hum. Reprod. 2020; 35: 2188–2196

Veldhuis, J.D., Urban, R.J., Lizarralde, G., Johnson, M.L., Iranmanesh, A. **Attenuation of luteinizing hormone secretory burst amplitude as a proximate basis for the hypoandrogenism of healthy aging in men.** J. Clin. Endocrinol. Metab. 1992; 75: 707–713

Veron, G.L., Tissera, A.D., Bello, R., Beltramone, F., Estofan, G., Molina, R.I., Vazquez-Levin, M.H. **Impact of age, clinical conditions, and lifestyle on routine semen parameters and sperm kinematics.** Fertil. Steril. 2018; 110

Whitcomb, B.W., Turzanski-Fortner, R., Richter, K.S., Kipersztok, S., Stillman, R.J., Levy, M.J., Levens, E.D. **Contribution of male age to outcomes in assisted reproductive technologies.** Fertil. Steril. 2011; 95: 147–151

Winkle, T., Rosenbusch, B., Gagsteiger, F., Paiss, T., Zoller, N. **The correlation between male age, sperm quality and sperm DNA fragmentation in 320 men attending a fertility center.** J. Assist. Reprod. Genet. 2009; 26: 41–46

Witkin, J.W. **Aging changes in synaptology of luteinizing hormone-releasing hormone neurons in male rat preoptic area.** Neuroscience 1987; 22: 1003–1013

Wyrobek, A.J., Eskenazi, B., Young, S., Arnheim, N., Tiemann-Boege, I., Jabs, E.W., Glaser, R.L., Pearson, F.S., Evenson, D. **Advancing age has differential effects on DNA damage, chromatin integrity, gene mutations, and aneuploidies in sperm.** Proc. Natl. Acad. Sci. U. S. A. 2006; 103: 9601–9606

Xie, D., Lu, C., Zhu, Y., Zhu, S., Yang, E.J., Jin, X. **Analysis on the association between sperm DNA fragmentation index and conventional semen parameters, blood microelements and seminal plasma ROS in male patients with infertility.** Exp. Ther. Med. 2018; 15: 5173–5176

Yuan, M., Huang, L., Leung, W.T., Wang, M., Meng, Y., Huang, Z., Pan, X., Zhou, J., Li, C., Sima, Y., Wang, L., Zhang, Y., Ying, C., Wang, L. **Sperm DNA fragmentation valued by SCSA and its correlation with conventional sperm parameters in male partner of recurrent spontaneous abortion couple.** Biosci. Trends 2019; 13: 152–159

Zorn, B., Vidmar, G., Meden-Vrtovec, H. **Seminal reactive oxygen species as predictors of fertilization, embryo quality and pregnancy rates after conventional in vitro fertilization and intracytoplasmic sperm injection.** Int. J. Androl. 2003; 26: 279–285

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