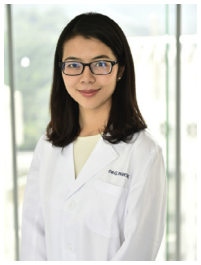
RBMO

ELSEVIER

ARTICLE

Comparison of DNA fragmentation levels in spermatozoa with different sex chromosome complements



BIOGRAPHY

Shi Xiao is a PhD student at the Chinese University of Hong Kong. Her research interest is male reproductive health. She was awarded an MD degree in 2012 from the Southern Medical University in China, and completed clinical training in reproductive medicine.

Xiao Shi, David Yiu Leung Chan, Ming Peng Zhao, Carol Pui Shan Chan, Jin Huang, Tin-Chiu Li*

KEY MESSAGE

The DNA fragmentation level of Y spermatozoa was significantly higher than X spermatozoa, it is also significantly higher in sex chromosome aneuploidy spermatozoa than the monosomic ones. These indicated a higher susceptibility to DNA damage in Y spermatozoa, an important cause of DNA damage which induced by the segregation errors.

ABSTRACT

Research question: Do spermatozoa with different sex chromosome complements (X and Y; aneuploidy and monosomy) exhibit different degrees of DNA damage?

Design: A prospective, observational study to measure the DNA fragmentation level and sex chromosome complement simultaneously using combined sperm chromosome dispersion (SCD) and fluorescence in-situ hybridization tests. Two methods were used to evaluate SCD images: a traditional semi-quantitative method to categorize halo size and a newly developed quantitative method based on the Matlab image analysis programme to more precisely measure the halo area and calculate the halo size index (HSI).

Results: The HSI (which was inversely proportional to DNA fragmentation level) of Y chromosome-bearing spermatozoa was significantly ($P < 0.05$) lower than that of X chromosome-bearing spermatozoa in both normozoospermic and pathozoospermic groups. The HSI of sex chromosome-aneuploid spermatozoa was also significantly ($P < 0.05$) lower than that of monosomic spermatozoa.

Conclusions: Our results indicated that Y chromosome-bearing spermatozoa are more susceptible to DNA damage than X chromosome-bearing spermatozoa, and the segregation errors during the meiotic division of spermatogenesis (resulting in aneuploidy) constitute an important contributory cause of DNA damage.

Assisted Conception Unit, Department of Obstetrics and Gynaecology, The Chinese University of Hong Kong, 1E, Special Block, Prince of Wales Hospital, Ngan Shing Street, Hong Kong

*Corresponding author. E-mail address: tinchiu.li@gmail.com (T-C Li). https://doi.org/10.1016/j.rbmo.2018.10.005
1472-6483/
Declaration: This study was funded by the Hong Kong Obstetrical and Gynaecological Trust Fund in 2017. The authors report no financial or commercial conflicts of interest.

KEYWORDS

DNA fragmentation
Fluorescence in-situ hybridization
Sex chromosome
Sperm chromatin dispersion

INTRODUCTION

Sperm DNA fragmentation, defined as the breakage of sperm DNA as a consequence of single or double strand breaks (*Robinson et al., 2012; Lewis et al. 2013*), is increasingly accepted as a supplementary test in infertility practice as previous studies demonstrated that sperm DNA fragmentation is correlated not only with classic semen parameters (*Stahl et al., 2015*) but also the likelihood and outcome of conception (*Simon et al., 2013; Osman et al., 2015; Carlini et al., 2017; Zheng et al., 2017*). Sperm DNA fragmentation can be induced by several mechanisms: apoptosis and strand breaks during the chromatin remodelling in the process of spermatogenesis; endogenous and exogenous reactive oxygen species (ROS); and radiotherapy, chemotherapy, and environmental factors such as air pollution (*Aitken and Krausz, 2001; Aitken and Koppers, 2011; Wright et al., 2014; Lafuente et al., 2016*). Moreover, the sources of sperm DNA fragmentation may also serve as potential causes of pathozoospermia. Accordingly, the sperm DNA fragmentation level in pathozoospermic samples was reported to be higher than that in normozoospermic samples (*Varghese et al., 2009*). It is not clear, however, whether spermatozoa with different sex chromosome complements have different susceptibility to DNA damage.

It has been long speculated that the Y chromosome is particularly vulnerable to DNA damage because DNA fragmentation in the Y chromosome is unable to be repaired by homologous recombination; instead, it can only be repaired by the non-homologous end-joining, a pathway that is more prone to error (*Aitken and Krausz, 2001; Kumar et al., 2013; Hatch, 2016*). Recently, *You et al. (2017)* found that the viability of Y chromosome-bearing spermatozoa was lower after exposure to different temperatures and culture periods than that of X chromosome-bearing spermatozoa, which indicated that the former survive less well under stressful conditions. It is still unknown, however, whether the DNA fragmentation levels in X and Y chromosome-bearing spermatozoa differ between normozoospermic and pathozoospermic groups.

Two recent studies have shown that the DNA fragmentation level and sperm aneuploidy rates were higher in subfertile men than in fertile controls (*Carrell et al., 2003; Perrin et al., 2011*). It has, therefore, been speculated that DNA fragmentation is somehow associated with sperm aneuploidy, including sex chromosome aneuploidy. Results from earlier studies, however, were controversial, with some studies showing sex chromosome-aneuploid spermatozoa as being associated with higher DNA fragmentation level (*Muriel et al., 2007; Enciso et al., 2013*), whereas others reported no significant correlation between sperm aneuploidy and DNA fragmentation (*Balasuriya et al., 2011*).

In the present study, we aimed to clarify whether spermatozoa with different sex chromosome complement (X and Y; aneuploidy and monosomy) have different DNA fragmentation levels. We simultaneously conducted sperm chromatin dispersion (SCD) and fluorescence in-situ hybridization (FISH) tests on the same semen samples from men with both normozoospermia and pathozoospermia. In addition, we measured SCD by two different methods: a traditional semi-quantitative method to categorize halo size and a newly developed quantitative and more objective method based on Matlab image analysis to measure the halo area precisely and calculate the halo size index (HSI).

MATERIALS AND METHODS

Semen samples

A total of 100 semen samples were collected from men who underwent semen analysis as part of the investigations for infertility at the IVF unit of Prince of Wales Hospital, the Chinese University of Hong Kong, between November 2015 and June 2016. Patients with azoospermia and genetic disorder were excluded from this study. All semen samples were obtained in hospital by masturbation after 3–7 days of sexual abstinence. Routine semen analysis was conducted, in addition to SCD testing and a combined SCD and FISH (SCD–FISH) test research procedure that was approved by the Joint Chinese University of Hong Kong – New Territories East Cluster Clinical Research Ethics Committee (REC No.: 2015.491) on 9 October 2015. To minimize the artefacts from induced DNA damage in the semen samples, extra pipetting and centrifugation were avoided in all experimental procedures (*Shi et al., 2016*), and semen samples were stored at –80°C immediately after semen analysis to avoid the ROS generation.

Routine semen analysis

Semen analysis was carried out manually according to World Health Organization guidelines (version V) (*WHO, 2010*). The measurement process conformed to the checklist recommended by *Björndahl et al. (2016)*. Briefly, after semen collection, samples were liquefied at 37°C and analysed within 1 h after ejaculation. After liquefaction, semen volume was measured using a wide-bore graduated pipette with a graduation of 0.1 ml. Sperm concentration was measured, and motility was assessed under a phase contrast microscope (OLYMPUS BX43, Tokyo, Japan) at a magnification of × 200. When measuring the sperm concentration, standard dilutions were used when necessary, and counting was carried out after 10–15-min sedimentation by haemocytometers with improved Neubauer ruling. When assessing sperm motility, a wet preparation was made with a 10-μl drop of semen sample and a 22 × 22 mm coverglass to give a depth of 20 μm. Duplicate assessments were made, and at least 200 spermatozoa were assessed for each sample. To evaluate sperm morphology, Tygerberg Strict Criteria (*Menkveld, 2013*) were used after staining the slides with a Diff-Quik staining kit (Dade Behring AG, Düdingen, Switzerland), and assessments were carried out under a microscope with an oil immersion × 100 objective (OLYMPUS BX43, Tokyo, Japan). Our laboratory participated in the external quality control scheme of the United Kingdom National External Quality Assessment Service (UK NEQAS).

Sperm samples were considered to be 'normal' when all parameters met the WHO reference values: volume 1.5 ml or over, concentration 15 × 10^6/ml or over, motility 40% or over, and morphology 4% or over (*WHO, 2010*). Samples with one or more abnormal semen parameters were classified as abnormal. Among the 100 semen samples selected for inclusion in the study, 50 samples had normal semen parameters (normozoospermic group), whereas the other 50 samples had one or more abnormal semen parameters

(pathozoospermic group). All semen analyses were conducted by one experienced technician who was blinded to the study.

SCD–FISH test

A modified SCD–FISH test, which combined the SCD and FISH test, was carried out according to the protocol described by *Fernández et al. (2011)* and *Muriel et al. (2007)*. The SCD test was conducted using a Halosperm G2 kit (Halotech, Madrid, Spain). Briefly, the semen samples were first diluted to a concentration less than 30 × 10^6/ml and mixed with melted agarose. Then, 8-µl of the mixture was dropped on a super-coated slide provided by the kit and a cover glass paced on top. After incubating at 4°C for 5 min, the cover glass was removed, and the slide was immersed in denature solution (solution 1 in the Halosperm G2 kit) for 7 min. Then, the slide was subsequently incubated in lysis solution (solution 2 in the Halosperm G2 kit) at room temperature (about 25°C) for 25 min. Following a 5-min wash procedure with double-distilled water, the slide was dehydrated in an ethanol series bath (70%, 90%, 100%), air-dried, and stored in a tightly closed box in the dark at room temperature until FISH was carried out.

When carrying out FISH, the slide was first incubated in 10% formaldehyde for 12 min. After washing by phosphate buffered saline, the slide was denatured in NaOH 0.05M/50% ethanol for 20 s, and dehydrated in an ethanol series and air-dried. Then, incubation was carried out overnight with a mixture of denatured DNA probes for alphoid centromeric regions of the X chromosome (DXZ1 Locus, SpectrumGreen; VysisInc Inc., Izasa, Spain) and Y chromosome (DYZ3 locus, SpectrumOrange; VysisInc Inc., Izasa, Spain) at 44°C. The slides were then washed in 50% formamide/2 × SSC, pH7, 44°C, for 8 min, and in 2 × SSC, pH7, 44°C, for 5 min. After cell counterstaining with 2 µg/ml DAPI (Sigma-Aldrich, St. Louis, MO, USA), the slide was then viewed under a Leica fluorescence microscope (Leica Microsystem DFC450, Nussloch, Germany.), which comprised bandpass filters with monochrome filters for DAPI (4′,6-diamidino-2-phenylindole), SpectrumGreen and SpectrumOrange. Images of each slide were taken using a high sensitivity charge coupled device camera under the same setting.

Spermatozoa with red or green fluorescent signals in the core area were considered as Y or X chromosome-bearing spermatozoa, respectively, and spermatozoa that showed more than one positive signal in the core area were considered as spermatozoa with sex chromosome aneuploidy (FIGURE 1). The data of sex chromosome complement were cross-checked by two different investigators.

SCD-only test

To estimate whether the FISH procedure may have affected the evaluation of DNA fragmentation level in the SCD–FISH test, five randomly selected samples underwent both the SCD-only test and the SCD–FISH test, and the results were compared. The procedure of the SCD-only test was the same as described above. Instead of performing FISH, the slides were stained by 2 µg/ml DAPI directly after the SCD procedure and then viewed under a fluorescence microscope (*Evenson 2016; Pratap et al., 2017*).

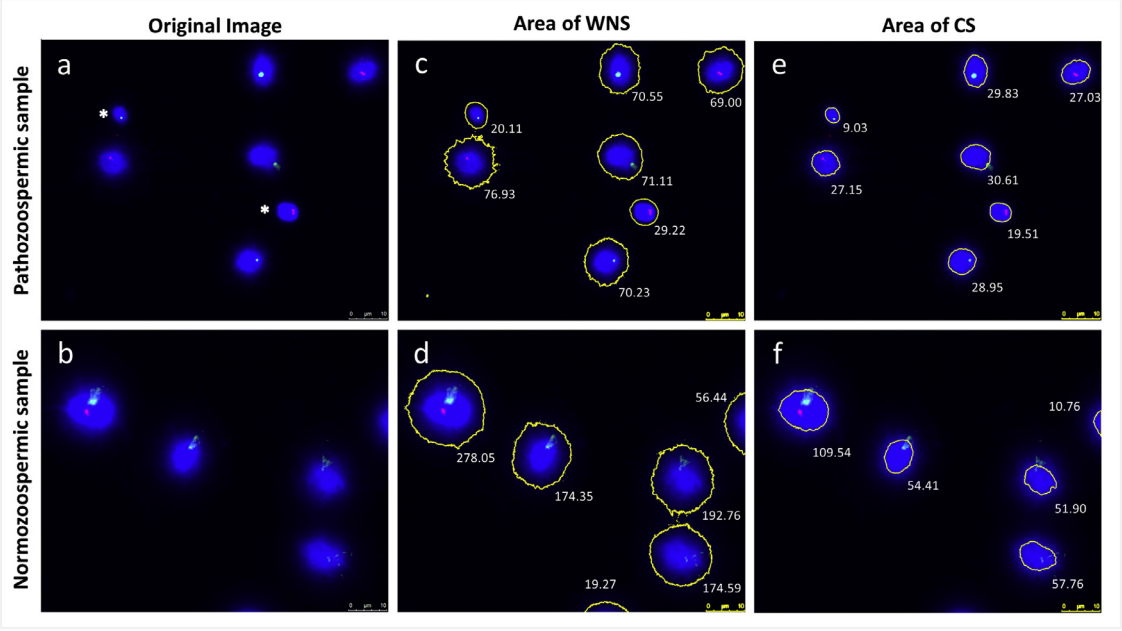


FIGURE 1 Two representative photo micrographs of the use of two methods (DNA fragmentation index [DFI] and halo size index [HIS]) to measure sperm chromatin dispersion, one from a pathozoospermic and the other from a normozoospermic sample. 1a and 1b: traditional assessment of halo size to determine DFI (spermatozoa with small or no halo per total number of spermatozoa assessed giving rise to DFI). *, Spermatozoa with small or no halo; 1c and 1d: use of image analysis techniques to measure the whole nuclear surface (WNS); 1e and 1f: use of image analysis techniques to measure the core surface (CS). The HSI is calculated as (WNS – CS) ÷ WNS. In the examples shown, 1a, 1c, and 1e are from the same sample of a man with pathozoospermia; 1b, 1d and 1f are from the same sample of a man with normozoospermia. On the basis of over 200 spermatozoa assessed from the same sample, the final DFI of 1a and 1b was 34.22% and 12.12%, respectively; the final HSI for 1c and 1e (pathozoospermia) was 52.26% and for 1d and 1f (normozoospermia) was 67.14%.

Traditional SCD evaluation method

The DNA fragmentation status of each spermatozoon was determined according to criteria similar to those of *Fernández et al. (2005b)*. Spermatozoa were classified into four patterns: spermatozoon with large halos (the halo width/minor diameter of the core was 1 or more); spermatozoon with medium-sized halos (halo size between that of the large and small halos); spermatozoon with small-sized halo (halo width/minor diameter of the core ≤1/3); and spermatozoon without a halo. Spermatozoa with small or no halo were considered as DNA-fragmented spermatozoa. The sperm DNA fragmentation calculated as the percentage of DNA fragmented spermatozoa among the total counted spermatozoa. According to a previous study (*Fernández et al., 2005b*), at least 200 spermatozoa (around 50 images) were evaluated for each sample, and only cells with a tail was considered as spermatozoa.

SCD measurement using Matlab Image Analysis Programme

An image analysis programme was developed on the basis of Matlab software to quantify the halo size objectively, derived by subtracting the core surface (CS) from the area of the whole nuclear surface (WNS) (FIGURE 1). HSI was calculated as the percentage of halo size (HS) (WNS minus CS) divided by WNS for each spermatozoon (*Fernández et al., 2003*), which is inversely proportional to the DNA fragmentation level.

The algorithm of our SCD image analysis programme is as follows: in the original SCD image, each pixel was superimposed by the three primary colours (red, green, blue) with different colour intensity, and the intensity of each primary colour of all pixels in the images was stored in three different channels (R, B, and G channel). As the sperm halos were stained using a blue fluorescent dye (DAPI), only light intensity stored in channel B was analysed. The original images were then converted to a greyscale image based on the light intensity in channel B, and the intensity of each pixel was transferred to a fractional value within a range between 0 (total absence, black) and 1 (total presence, white). The boundary of WNS was identified by comparing the pixel intensity and a threshold 'M'. If the intensity of a pixel was smaller than M, this pixel was considered outside the WNS and vice versa. In order to distinguish the boundary of WNS, the pixel intensity less than M was set to zero, and the greyscale image was converted to a binary image according to Otsu's method (*Chen et al., 2012*). In this manner, the image of WNS was converted to a white block and its boundary could be distinguished, and highlighted, and the area calculated. The area of core surface could be obtained in the same way. The setting of threshold M was based on the testing of over 100 images, and it was confirmed after three observers reached an agreement. Once M was agreed, all images were analysed using the same threshold.

After the programme analysis, only the data from spermatozoa with clear and single halos were collected. Sperm images were excluded from analysis if meet the following reasons: only part of the halo was visualized; if the halo from one spermatozoon overlapped with another; or debris was present that produced interference (FIGURE 2). Among all SCD–FISH images, the data of WNS and CS from 80 samples were used for final analysis, whereas images from 20 samples were excluded because of the relatively high sperm density, which reduced the number of sperm suitable for analysis to less than 200. The data collection was cross-checked by two different investigators.

Inter-observer and intra-observer variability of the two SCD evaluation methods

The inter- and intra- observer variability for DFI and HSI were measured by assessing images from 200 randomly chosen spermatozoa. Kappa values were calculated to compare the agreement level of different observers and evaluations. In calculating the kappa value for HSI measurement, the results were converted to one of four categorical values: large, medium, small, and no halo, equivalent to less than 25th, more than 25th or and less than 50th, more than 50th and less than 75th, and 75th and over percentile of HSI results based on the total population of spermatozoa examined (n = 17,712) from the 80 samples.

Statistical analysis

SPSS 20.0 package software (IBM Corp., USA) was used for all data analysis. Mann–Whitney U test was used to compare the baseline characteristics

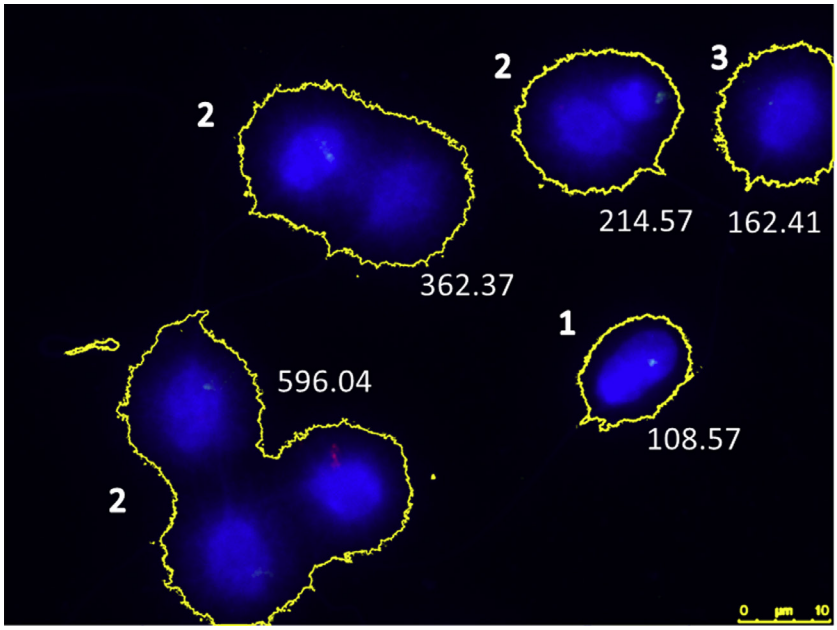


FIGURE 2 Representative photo micrograph of included and excluded samples in halo size index measurement. The values shown refer to the area of the whole nuclear surface (WNS). (1) a spermatozoon in which the WNS boundary was properly identified and so suitable for inclusion; (2) spermatozoa that were too close together, which precluded the individual boundary of the WNS to be defined; the WNS as measured showed the aggregate results. Such a finding is more likely to be encountered in samples with high density. They were not suitable for inclusion; (3) a spermatozoon with part of the halo outside the microscopic field; the measurement (WNS area 162.41) represented underestimation and so should be excluded.

TABLE 1 COMPARISON OF BASELINE CHARACTERISTIC AND SEMEN PARAMETERS OF THE TWO STUDY POPULATIONS

Characteristic	Normozoospermic group	Pathozoospermic group	*P*-value
Age (years)[a]	37.0 (33.8–42.0)	39.0 (25.0–44.3)	NS
Body mass index (kg/m^2)[a]	23.8 (21.6–27.0)	24.8 (22.5–27.9)	NS
Abstinence time (days)[a]	4.0 (3.0–4.3)	4.0 (3.0–4.3)	NS
Ejaculation volume (ml)[a]	2.5 (2.0–4.0)	2.8 (2.0–3.5)	NS
Sperm concentration (million/ml)[a]	50.0 (37.0–83.5)	26.5 (12.0–52.0)	<0.001
% Motile sperm[a]	54.0 (47.0–62.3)	35.5 (25.8–45.0)	<0.001
% Normal morphology[a]	4.5 (4.0–5.0)	2.0 (1.0–3.5)	<0.001
% Sex chromosome aneuploidy[a]	2.4 (1.8–4.8)	4.8 (2.7–8.9)	0.01
DFI (%)[a]	18.7 (11.7–26.1)	24.9 (20.3–29.2)	<0.001
HSI (%)[b]	66.8 (62.4–69.7)	61.6 (57.6–64.4)	<0.001

[a] Fifty participants in each group;

[b] Forty participants in each group.

DFI, DNA Fragmentation Index; HSI, halo size index; NS, non-significant.

and DNA fragmentation level (DFI and HSI) between different groups and different sex chromosome complements. Kappa value was calculated to compare the inter- and intra- observer variability of the two SCD evaluation methods. The correlations between DNA fragmentation level and classic semen parameters were calculated using the Spearmen correlation coefficient. $P < 0.05$ was considered to indicate significance.

RESULTS

Baseline characteristics and semen parameters of participants

One hundred subjects were recruited in this study, including 50 men with normozoospermia (normozoospermic group) and 50 men with pathozoospermia (pathozoospermic group). Seven phenotypes were identified in the pathozoospermic group: oligospermia ($n = 2$), asthenozoospermia ($n = 7$), teratozoospermia ($n = 15$), oligoasthenozoospermia ($n = 1$), oligoteratozoospermia ($n = 4$), asthenoteratozoospermia ($n = 11$) and oligoasthenoterazoospermia ($n = 10$). An illustration of DNA fragmentation level and sex chromosome complement as visualized by the SCD–FISH test and the processed results of the SCD image analysis programme, are presented in FIGURE 1. From the 100 semen samples obtained, a total of 31,512 spermatozoa were counted to calculate the sperm DFI (approximately 300 spermatozoa per sample), and the HSI of 17,712 spermatozoa from 80 samples with suitable sperm density were calculated (about 200 spermatozoa per sample). In the HSI analysis, 20 samples were excluded because of the relatively high sperm density in the images, which resulted in frequent overlapping of halo boundaries (FIGURE 2), reducing the number of analysable spermatozoa to less than 200. The demographics and semen parameters of the normozoospermic and pathozoospermic groups are compared in TABLE 1. No significant difference was detected in age, body mass index (BMI), abstinence time and ejaculation volume between the two groups. In the pathozoospermic group, the sperm concentration, motility, morphology and HSI were significantly lower ($P < 0.05$), whereas DFI and sex chromosome aneuploidy rate were significantly higher ($P < 0.05$) than those in the normozoospermic group. No significant difference was found in demographic and semen parameters between participants with or without HSI measurement (Supplementary TABLE 1).

Inter- and intra-observer variability of DFI and HSI

The kappa value of intra-observer variability of DFI was as follows: observer A = 0.57 (agreement percentage = 72.5%); and observer B = 0.46 (agreement percentage = 62%); mean of observer A and B = 0.52 (mean agreement percentage = 67%). The kappa value of inter-observer variability of DFI was 0.38 (agreement percentage = 55%). The kappa value of intra- and inter-observer variability of HSI (when categorized into four size groups, see definition above) was 1 (agreement percentage = 100%).

The effect of FISH testing on HSI

The mean HSI measured by SCD–FISH was 62.0%, which did not significantly differ from that of the SCD-only test (60.8%).

Comparison between X and Y Chromosome-bearing spermatozoa

The results of DFI and HSI in X chromosome-bearing spermatozoa are compared with those of Y chromosome-bearing spermatozoa in TABLE 2 and illustrated in FIGURE 3 (a–f). In the normozoospermic group, the DFI of Y chromosome-bearing spermatozoa did not significantly differ from that of X chromosome-bearing spermatozoa, whereas in the pathozoospermic group the DFI of Y chromosome-bearing spermatozoa was significantly ($P = 0.04$) higher than that of X chromosome-bearing spermatozoa. When both groups were considered together, the DFI of Y chromosome-bearing spermatozoa was significantly higher ($P = 0.02$) than that of X chromosome-bearing spermatozoa. In contrast, the HSI of Y chromosome-bearing spermatozoa was significantly lower than that of X chromosome-bearing spermatozoa in both normozoospermic ($P = 0.01$) and pathozoospermic groups ($P = 0.03$) as well as when the two groups were considered together ($P = 0.001$).

Comparison between sex chromosome monosomic and aneuploid spermatozoa

The comparisons of DFI and HSI in sex chromosome monosomic and sex chromosome aneuploid spermatozoa are presented in TABLE 3

TABLE 2 COMPARISONS OF DNA FRAGMENTATION INDEX AND HALO SIZE INDEX BETWEEN X AND Y CHROMOSOME-BEARING SPERMATOZOA, SEPARATELY FOR THE NORMOZOOSPERMIC, PATHOZOOSPERMIC GROUP AND FOR BOTH GROUPS COMBINED

DNA fragmentation	Chromosome-bearing sperm	Normozoospermic group		Pathozoospermic group		Two groups combined	
		Counted sperm	Median (IQR)	Counted sperm	Median (IQR)	Counted sperm	Median (IQR)
DFI (%)[a]	X	7297	17.3 (11.3–25.2)	7564	22.5 (18.4–29.8)	14861	20.4 (12.9–28.3)
	Y	7345	17.2 (11.4–29.3)	7736	25.9 (18.8–32.9)	15081	23.5 (15.3–30.8)
	P-Value		NS		0.04		0.02
HSI (%)[b]	X	4235	67.8 (63.3–70.9)	4849	62.9 (59.2–64.9)	9084	64.5 (60.4–68.5)
	Y	3696	65.6 (61.6–70.0)	4390	61.9 (56.7–64.7)	8086	63.0 (58.9–67.9)
	P Value		0.01		0.03		0.001

[a] Fifty participants in both normozoospermic and pathozoospermic groups.

[b] Forty participants in both normozoospermic and pathozoospermic groups.

DFI, DNA fragmentation index; HSI, halo size index; IQR, interquartile range; NS, not statistically significant.

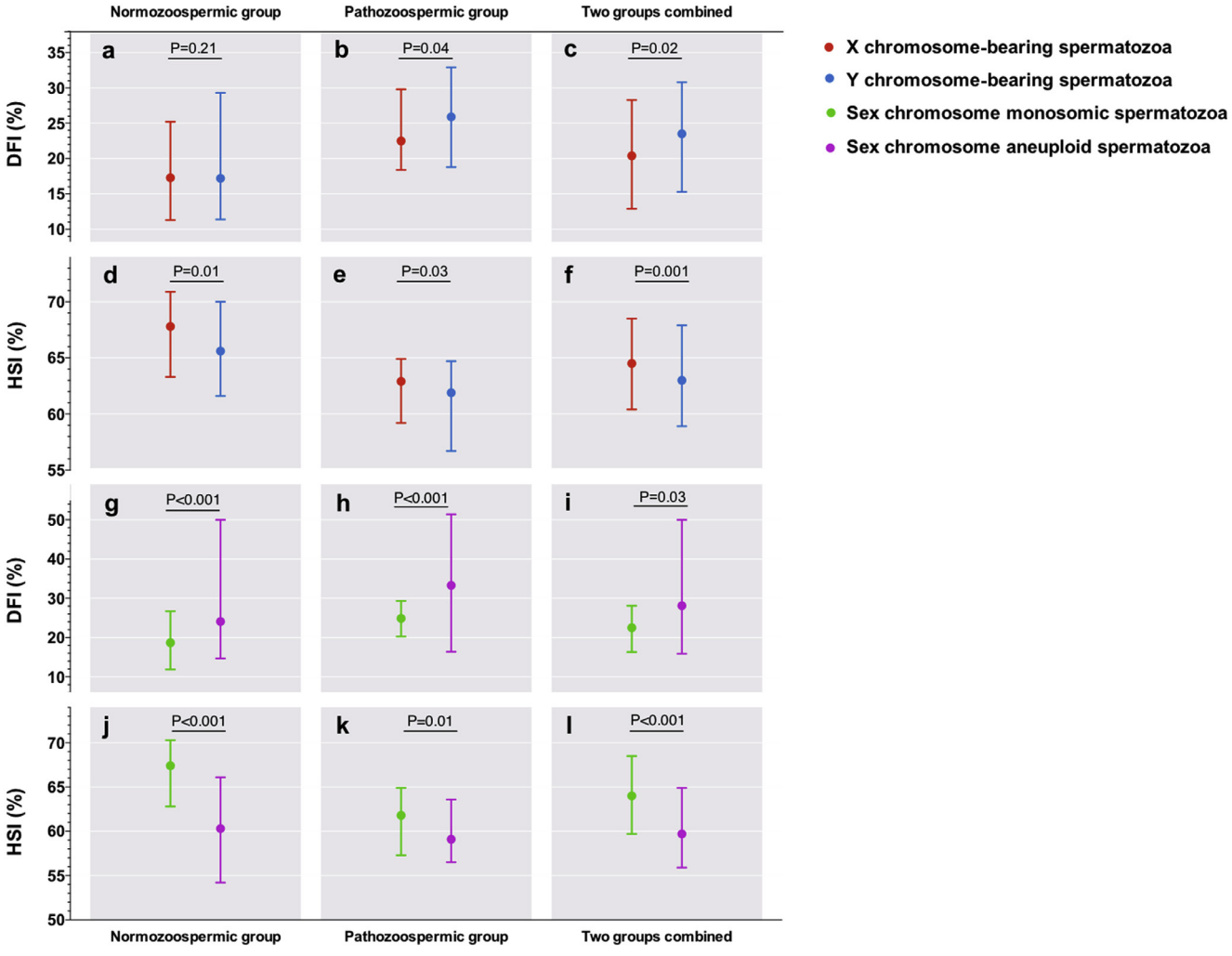


FIGURE 3 Comparisons of DNA fragmentation index (DFI) between X and Y chromosome-bearing spermatozoa (a–c), halo size index (HSI) between X and Y chromosome-bearing spermatozoa (d–f), DFI between aneuploid and monosomic sex chromosome spermatozoa (g–i), and HSI between aneuploid and monosomic sex chromosome spermatozoa (j–i) in normozoospermic, pathozoospermic, and both groups combined, respectively. The dots, and upper and lower horizontal lines represent the median, 25% and 75% quantile, respectively. The red and blue dots represent X and Y chromosome bearing spermatozoa, respectively. The green and purple dots represent monosomic and aneuploid sex chromosome spermatozoa, respectively.

TABLE 3 COMPARISONS OF DNA FRAGMENTATION INDEX AND HALO SIZE INDEX BETWEEN SEX CHROMOSOME ANEUPLOID SPERMATOZOA AND SEX CHROMOSOME MONOSOMIC SPERMATOZOA, SEPARATELY FOR THE NORMOZOOSPERMIC AND PATHOZOOSPERMIC GROUPS, AND FOR BOTH GROUPS COMBINED

DNA fragmentation	Chromosome-bearing sperm	Normozoospermic group		Pathozoospermic group		Two groups combined	
		Counted sperm	Median (IQR)	Counted sperm	Median (IQR)	Counted sperm	Median (IQR)
DFI (%)[a]	Sex chromosome monosomy	14642	18.7 (11.9–26.7)	15300	24.9 (20.3–29.3)	29942	22.5 (16.3–28.1)
	Sex chromosome aneuploidy	635	24.1 (14.7–50.0)	935	33.3 (16.4–51.4)	1570	28.1 (15.9–50.0)
	P-Value		< 0.001		< 0.001		0.03
HSI (%)[b]	Sex chromosome monosomy	7931	67.4 (62.8–70.3)	9239	61.8 (57.3–64.9)	17170	64.0 (59.7–68.5)
	Sex chromosome aneuploidy	238	60.3 (54.2–66.1)	304	59.1 (56.5–63.6)	542	59.7 (55.9–64.9)
	P-Value		< 0.001		0.01		< 0.001

[a] Fifty participants in the normozoospermic and pathozoospermic group.

[b] Forty participants in the normozoospermic and pathozoospermic group.

DFI, DNA fragmentation index; HSI, halo size index; IQR, interquartile range.

and FIGURE 3 (g–l). The DFI of sex chromosome aneuploid spermatozoa was significantly higher than that of sex chromosome monosomic spermatozoa in the normozoospermic ($P < 0.001$), pathozoospermic ($P < 0.001$) and combined groups ($P = 0.03$). Conversely, the HSI of sex chromosome aneuploid spermatozoa was significantly lower than that of sex chromosome monosomic spermatozoa in the normozoospermic ($P < 0.001$), pathozoospermic ($P = 0.01$) and the combined groups ($P < 0.001$).

Comparison of DFI and HSI between normozoospermic and pathozoospermic groups

In the pathozoospermic group, the DFI of sex chromosome monosomic spermatozoa was significantly higher ($P = 0.007$) and HSI was significantly lower ($P < 0.001$) than those of the normozoospermic group. No significant difference in DFI or HSI of sex chromosome aneuploidy was observed between the normozoospermic and pathozoospermic groups.

Correlation between DFI and HSI and demographic and semen parameters

A negative correlation was found between DFI and sperm motility ($R = -0.38$; $P < 0.001$) and morphology ($R = -0.38$; $P < 0.001$), whereas HSI was positively correlated ($R = 0.40$; $P < 0.001$ and $R = 0.39$; $P < 0.001$, respectively). No significant correlation, however, was observed between DFI and age, BMI, semen volume and sperm concentration, or between HSI and age, BMI, semen volume, and sperm concentration. The dot plot graphs of the correlation between DFI/HSI and demographic and semen parameters are presented in Supplementary FIGURE 1 and Supplementary FIGURE 2.

DISCUSSION

In this study, the DNA fragmentation level was compared between spermatozoa with different sex chromosome complements by two different methods: a traditional semi-quantitative method (DFI) and a newly developed quantitative method (HSI). To our knowledge, this is the first study investigating the relationship between different sex chromosome complement and sperm DNA fragmentation level. Notably, our study identified several significant findings.

First, we found that, in the pathozoospermic group, the DNA fragmentation level as measured by both DFI and HSI in Y chromosome-bearing spermatozoa was significantly higher than that in the X chromosome-bearing spermatozoa. In the normozoospermic group, however, the two methods of measurement produced discordant results. According to the semi-quantitative method (DFI), no significant difference was identified between X and Y chromosome-bearing spermatozoa, whereas, according to the quantitative method (HSI) using an image capture and analysis programme, the DNA fragmentation level of Y chromosome-bearing spermatozoa was found to be significantly higher than that of X chromosome-bearing spermatozoa, as in the case of the pathozoospermic group. We consider that the results obtained by HSI are more likely to be reliable as it is a quantitative method, being more precise and more reproducible as shown by the high kappa value of both intra- and inter- observer variability compared with the medium to low kappa value of the semi-quantitative DFI method. Furthermore, when the two sample groups (pathozoospermic and normozoospermic groups) were considered together, the overall result also showed that the DNA fragmentation level as measured by both DFI and HSI methods in Y chromosome-bearing spermatozoa was significantly higher than that of X chromosome-bearing spermatozoa. Taken together, it seems likely that Y chromosome-bearing spermatozoa are more susceptible to DNA fragmentation than X chromosome-bearing spermatozoa. In comparison, in an earlier study in mice, it was found that after irradiation damage, DNA fragmentation levels increased significantly and the percentage of zona pellucida binding Y chromosome-bearing spermatozoa was significantly lower than that of X chromosome-bearing spermatozoa, suggesting that Y chromosome-bearing spermatozoa are relatively less tolerant of irradiation damage (*Kumar et al.*,

2013). In the study by Kumar et al. (2013), however, no attempt was made to examine the difference in DNA fragmentation rate between X and Y chromosome-bearing spermatozoa. Interestingly, You et al. (2017) found that the Y chromosome-bearing spermatozoa are more vulnerable to fluctuations in physiological and sperm-storage conditions than X chromosome-bearing spermatozoa, which may be due to a weaker defence system of Y chromosome-bearing spermatozoa against oxidative stress.

Nevertheless, the clinical relevance of such a finding is not certain. If the difference in DNA fragmentation level between X and Y chromosome-bearing spermatozoa has a profound clinical effect on fertilization rate or normal embryo development, it would be expected that a significant association between birth gender ratio in favour of girls in patients with higher DNA fragmentation would be observed. Although some recent studies have demonstrated that exposure to environmental toxins, such as endocrine disruptors and pollutants, is associated with higher sperm DNA fragmentation level, lower sperm Y:X chromosome ratios and lower male birth rate (Mocarelli et al., 2000; del Rio Gomez et al., 2002; Robbins et al. 2008; Kvist et al., 2012; 2014), no firm data suggest that increased DNA fragmentation relates to the female : male sex ratio at birth.

Second, we found that the DNA fragmentation level (measured by DFI and HSI) of spermatozoa with sex chromosome aneuploidy was significantly higher than that of spermatozoa with monosomic sex chromosome (X or Y) in both normozoospermic and pathozoospermic groups. This result indicated that spermatozoa with sex chromosome aneuploidy have a higher DNA fragmentation level. In comparison, when only sex chromosome monosomic spermatozoa were considered, the DNA fragmentation level (measured by both DFI and HSI) in the pathozoospermic group was higher than that in the normozoospermic group; however, when only sex chromosome aneuploid spermatozoa were considered, we found no significant difference in DFI and HSI between spermatozoa derived from the normozoospermic and pathozoospermic groups. The testicular environment, such as endogenous and exogenous ROS and other environmental or iatrogenic factors, is considered an important contributory factor for pathozoospermia, and may also represent a potential cause of sperm DNA fragmentation (Muriel et al., 2007; Enciso et al., 2013; McAuliffe et al., 2014). This may also be the reason for the higher DNA fragmentation level of sex chromosome monosomic spermatozoa in the pathozoospermic group. Alternatively, the sperm aneuploidy is mainly caused by segregation errors during the meiotic division, which is another source of DNA fragmentation (McAuliffe et al., 2014). Together, our findings suggest that segregation errors during the meiotic division play a relatively more dominant role in inducing the DNA damage than the testicular environment. Moreover, it has also been recently demonstrated that a high level of DNA fragmentation could itself lead to uneven distribution between daughter cells during mitosis, in turn resulting in subsequent segregation errors (Zhang et al., 2015).

A particular strength of our study is that we used both traditional semi-quantitative SCD evaluation method (DFI) and quantitative SCD image analysis programme to measure the DNA fragmentation level of each spermatozoon, which permitted a direct comparison of the two methods. The two methods produced similar results in most, but not all, the comparisons, which highlighted the importance of applying the quantitative and more precise method in the study of sperm DNA fragmentation using the SCD method. The limited precision of the traditional semi-quantitative method may thus constitute a source of the reported controversies regarding the prognostic value and clinical usefulness of SCD testing (Cissen et al., 2016; Esteves et al., 2017). A reappraisal of the value of sperm DNA fragmentation testing by applying a quantitative and precise method, therefore, seems to be justified. In our study, the number of spermatozoa examined using the traditional semi-quantitative SCD evaluation method was higher than those evaluated using the quantitative SCD image analysis programme (TABLE 2 and TABLE 3), in part because of the stricter requirement of the latter, which excluded spermatozoa with heads overlapping with one another, and partly because when we obtained the original images for traditional semi-quantitative SCD evaluation, we subconsciously kept images with a higher density and did not appreciate the strict requirement regarding overlapping spermatozoa in the quantitative SCD image analysis programme, which was developed in response to the suggestion of the journal reviewer. With the benefit of hindsight, we should have kept more images with lower sperm density per field.

Several methods have been proposed to measure sperm DNA fragmentation level: these include sperm chromatin structure assay, terminal uridine nick-end labelling (TUNEL), comet assay and SCD. Among these four available methods, we chose SCD for several reasons. First, only SCD and TUNEL could be carried out together with the use of FISH (Muriel et al., 2007). Second, in choosing between SCD and TUNEL, we preferred SCD because it is considered to have higher sensitivity and better reproducibility than the TUNEL test (Fernández et al., 2005a; 2005b; Zhang et al., 2010). Furthermore, with the benefit of hindsight, we have been able to further improve the precision of the SCD test by using image analysis techniques. Using the SCD–FISH method, we were able to directly observe DNA fragmentation status and chromosome complement in each single spermatozoon at the same time (Enciso et al., 2006; Muriel et al. 2007; Balasuriya et al., 2011; McAuliffe et al. 2014) and demonstrated that the concurrent use of FISH did not significantly alter the result of the SCD test (HSI), consistent with the findings of Enciso et al. (2006). Finally, our result showed that the DFI and HSI were both significantly correlated with the sperm motility and morphology, which was in line with the finding of a previous study (Boushaba and Belaaloui, 2015). Our study, however, is limited to the effect of sex chromosome aneuploidy on DNA fragmentation rate, as we did not have information regarding the autosomes of the spermatozoa.

In conclusion, we have shown that the use of a modified, quantified SCD method in the study of sperm DNA fragmentation provides more precise, reliable results than the conventional semi-quantitative method. More importantly, based on these analyses, we found that a higher level of DNA fragmentation occurred in Y chromosome-bearing spermatozoa than

X chromosome-bearing spermatozoa, and a higher level of DNA fragmentation occurred in aneuploid sex chromosome spermatozoa than monosomic sex chromosome spermatozoa. The functional relevance of the findings requires, however, further investigation.

ACKNOWLEDGEMENTS

We are grateful to Professor Christopher Barratt of the University of Dundee (Honorary Professor of the Chinese University of Hong Kong) for his advice on the manuscript, Dr Yvonne Ka-Yin Kwok of the Chinese University of Hong Kong for her assistance with the experimental protocol, and all the participants who agreed to take part in this study.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.rbmo.2018.10.005.

REFERENCES

Aitken, R.J., Koppers, A.J. **Apoptosis and DNA damage in human spermatozoa.** Asian J Androl 2011; 13: 36–42

Aitken, R.J., Krausz, C. **Oxidative stress, DNA damage and the Y chromosome.** Reproduction 2001; 122: 497–506

Balasuriya, A., Speyer, B., Serhal, P., Doshi, A., Harper, J.C. **Sperm chromatin dispersion test in the assessment of DNA fragmentation and aneuploidy in human spermatozoa.** Reprod Biomed Online 2011; 22: 428–436

Björndahl, L., Barratt, C.L., Mortimer, D., Jouannet, P. **'How to count sperm properly': checklist for acceptability of studies based on human semen analysis.** Hum Reprod 2016; 31: 227–232

Boushaba, S., Belaaloui, G. **Sperm DNA fragmentation and standard semen parameters in algerian infertile male partners.** World J Mens Health 2015; 33: 1–7

Carlini, T., Paoli, D., Pelloni, M., Faja, F., Dal Lago, A., Lombardo, F., Lenzi, A., Gandini, L. **Sperm DNA fragmentation in Italian couples with recurrent pregnancy loss.** Reprod Biomed Online 2017; 34: 58–65

Carrell, D.T., Wilcox, A.L., Lowy, L., Peterson, C.M., Jones, K.P., Erickson, L., Campbell, B., Branch, D.W., Hatasaka, H.H. **Elevated sperm chromosome aneuploidy and apoptosis in patients with unexplained recurrent pregnancy loss.** Obstet Gynecol 2003; 101: 1229–1235

Chen, Q., Zhao, L., Lu, J., Kuang, G., Wang, N., Jiang, Y. **Modified two-dimensional Otsu image segmentation algorithm and fast realisation.** IET Image Processing 2012; 6: 426–433

Cissen, M., Wely, M.V., Scholten, I., Mansell, S., Bruin, J.P., Mol, B.W., Braat, D., Repping, S., Hamer, G. **Measuring sperm DNA fragmentation and clinical outcomes of medically assisted reproduction: A Systematic Review and Meta-Analysis.** PLoS One 2016; 11e0165125

del Rio Gomez, I., Marshall, T., Tsai, P., Shao, Y.S., Guo, Y.L. **Number of boys born to men exposed to polychlorinated byphenyls.** Lancet 2002; 360: 143–144

Enciso, M., Alfarawati, S., Wells., D. **Increased numbers of DNA-damaged spermatozoa in samples presenting an elevated rate of numerical chromosome abnormalities.** Hum Reprod 2013; 28: 1707–1715

Enciso, M., López-Fernández, C., Fernández, J.L., Garcia, P., Gosálbez, A., Gosálvez, J. **A new method to analyze boar sperm DNA fragmentation under bright-field or fluorescence microscopy.** Theriogenology 2006; 65: 308–316

Esteves, S.C., Roque, M., Bradley, C.K., Garrido, N. **Reproductive outcomes of testicular versus ejaculated sperm for intracytoplasmic sperm injection among men with high levels of DNA fragmentation in semen: systematic review and meta-analysis.** Fertil Steril 2017; 108: 456–467

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

Fernández, J.L., Muriel, L., Goyanes, V., Segrelles, E., Gosálvez, J., Enciso, M., LaFromboise, M., De Jonge, C. **Halosperm is an easy, available, and cost-effective alternative for determining sperm DNA fragmentation.** Fertil Steril 2005; 84: 860

Fernández, J.L., Muriel, L., Goyanes, V., Segrelles, E., Gosálvez, J., Enciso, M., LaFromboise, M., De Jonge, C. **Simple determination of human sperm DNA fragmentation with an improved sperm chromatin dispersion test.** Fertil Steril 2005; 84: 833–842

Fernández, J.L., Muriel, L., Rivero, M.T., Goyanes, V., Vazquez, R., Alvarez, J.G. **The sperm chromatin dispersion test: a simple method for the determination of sperm DNA fragmentation.** J Androl 2003; 24: 59–66

Fernández, J.L., Cajigal, D., López-Fernández, C., Gosálvez, J. **Assessing sperm DNA fragmentation with the sperm chromatin dispersion test.** Methods Mol Biol 2011; 682: 291–301

Hatch, E.M. **Y chromothripsis?.** Nat Cell Biol 2016; 19: 12–14

Kumar, D., Upadhya, D., Uppangala, S., Salian, S.R., Kalthur, G., Adiga, S.K. **Nuclear DNA fragmentation negatively affects zona binding competence of Y bearing mouse spermatozoa.** J Assist Reprod Genet 2013; 30: 1611–1615

Kvist, L., Giwercman, A., Weihe, P., Kold Jensen, T., Grandjean, P., Halling, J., Skaalum Petersen, M., Lundberg Giwercman, Y. **Exposure to persistent organic pollutants and sperm sex chromosome ratio in men from the Faroe Islands.** Environ Int 2014; 73: 359–364

Kvist, L., Giwercman, Y.L., Jönsson, B.A., Lindh, C.H., Bonde, J.P., Toft, G., Strucinski, P., Pedersen, H.S., Zvyezday, V., Giwercman, A. **Serum levels of perfluorinated compounds and sperm Y:X chromosome ratio in two European populations and in Inuit from Greenland.** Reprod Toxicol 2012; 34: 644–650

Lafuente, R., Garcia-Blàquez, N., Jacquemin, B., Checa, M.A. **Outdoor air pollution and sperm quality.** Fertil Steril 2016; 106: 880–896

Lewis, S.E., John Aitken, R., Conner, S.J., Iuliis, G.D., Evenson, D.P., Henkel, R., Giwercman, A., Gharagozloo, P. **The impact of sperm DNA damage in assisted conception and beyond: recent advances in diagnosis and treatment.** Reprod Biomed Online 2013; 27: 325–337

McAuliffe, M.E., Williams, P.L., Korrick, S.A., Dadd, R., Marchetti, F., Martenies, S.E., Perry, M.J. **Human sperm sex chromosome disomy and sperm DNA damage assessed by the neutral comet assay.** Hum Reprod 2014; 29: 2148–2155

Menkveld, R. **Sperm morphology assessment using strict (tygerberg) criteria.** Methods Mol Biol 2013; 927: 39–50

Mocarelli, P., Gerthoux, P.M., Ferrari, E., Patterson, D.G.Jr., Kieszak, S.M., Brambilla, P., Vincoli, N., Signorini, S., Tramacere, P., Carreri, V., Sampson, E.J., Turner, W.E., Needham, L.L. **Paternal concentrations of dioxin and sex ratio of offspring.** Lancet 2000; 355: 1858–1863

Muriel, L., Goyanes, V., Segrelles, E., Gosàlvez, J., Alvarez, J.G., Fernàndez, J.L. **Increased aneuploidy rate in sperm with fragmented DNA as determined by the sperm chromatin dispersion (SCD) test and FISH analysis.** J Androl 2007; 28: 38–49

Osman, A., Alsomait, H., Seshadri, S., El-Toukhy, T., Khalaf, Y. **The effect of sperm DNA fragmentation on live birth rate after IVF or ICSI: a systematic review and meta-analysis.** Reprod Biomed Online 2015; 30: 120–127

Perrin, A., Basinko, A., Douet-Guilbert, N., Gueganic, N., Le Bris, M.J., Amice, V., De Braekeleer, M., Morel, F. **Aneuploidy and DNA fragmentation in sperm of carriers of a constitutional chromosomal abnormality.** Cytogenet Genome Res 2011; 133: 100–106

Pratap, H., Hottigoudar, S.Y., Nichanahalli, K.S., Chand, P. **Assessment of Sperm Deoxyribose Nucleic Acid Fragmentation Using Sperm Chromatin Dispersion Assay.** J Pharmacol Pharmacother 2017; 8: 45–49

Robbins, W.A., Wei, F., Elashoff, D.A., Wu, G., Xun, L., Jia, J. **Y:X sperm ratio in boron-exposed men.** J Androl 2008; 29: 115–121

Robinson, L., Gallos, I.D., Conner, S.J., Rajkhowa, M., Miller, D., Lewis, S., Kirkman-Brown, J., Coomarasamy, A. **The effect of sperm DNA fragmentation on miscarriage rates: a systematic review and meta-analysis.** Hum Reprod 2012; 27: 2908–2917

Shi, X., Wang, T., Qiu, Z.L., Li, K., Li, L., Chan, C.P., Chan, S.M., Li, T.C., Quan, S. **Effects of mechanical stresses on sperm function and fertilization rate in mice.** Syst Biol Reprod Med 2016; 62: 152–159

Simon, L., Proutski, I., Stevenson, M., Jennings, D., McManus, J., Lutton, D., Lewis, S.E. **Sperm DNA damage has a negative association with live-birth rates after IVF.** Reprod Biomed Online 2013; 26: 68–78

Stahl, P.J., Cogan, C., Mehta, A., Bolyakov, A., Paduch, D.A., Goldstein, M. **Concordance among sperm deoxyribonucleic acid integrity assays and semen parameters.** Fertil Steril 2015; 104: 56–61

Varghese, A.C., Bragais, F.M., Mukhopadhyay, D., Kundu, S., Pal, M., Bhattacharyya, A.K., Agarwal, A. **Human sperm DNA integrity in normal and abnormal semen samples and its correlation with sperm characteristics.** Andrologia 2009; 41: 207–215

World Health Organisation. WHO Laboratory Manual for the Examination and Processing of Human Semen. 5th ed2010World Health OrganizationGeneva

Wright, C., Milne, S., Leeson, H. **Sperm DNA damage caused by oxidative stress: modifiable clinical, lifestyle and nutritional factors in male infertility.** Reprod Biomed Online 2014; 28: 684–703

You, Y.A., Kwon, W.S., Saidur Rahman, M., Park, Y.J., Kim, Y.J., Pang, M.G. **Sex chromosome-dependent differential viability of human spermatozoa during prolonged incubation.** Hum Reprod 2017; 32: 1183–1191

Zhang, C.Z., Spektor, A., Cornils, H., Francis, J.M., Jackson, E.K., Liu, S., Meyerson, M., Pellman, D. **Chromothripsis from DNA damage in micronuclei.** Nature 2015; 522: 179–184

Zhang, L.H., Qiu, Y., Wang, K.H., Wang, Q., Tao, G., Wang, L.G. **Measurement of sperm DNA fragmentation using bright-field microscopy: comparison between sperm chromatin dispersion test and terminal uridine nick-end labeling assay.** Fertil Steril 2010; 94: 1027–1032

Zheng, W.W., Song, G., Wang, Q.L., Liu, S.W., Zhu, X.L., Deng, S.M., Zhong, A., Tan, Y.M., Tan, Y. **Sperm DNA damage has a negative effect on early embryonic development following in vitro fertilization.** Asian J Androl 2017; 20: 75–79

Received 2 March 2018; received in revised form 17 October 2018; accepted 17 October 2018.