Received: 14 March 2019 | Revised: 15 April 2019 | Accepted: 26 April 2019

DOI: 10.1111/and.13316

ORIGINAL ARTICLE

First International Journal of Andrology aNDROLOGIa WILEY

Diagnostics of DNA fragmentation in human spermatozoa: Are sperm chromatin structure analysis and sperm chromatin dispersion tests (SCD-HaloSpermG2®) comparable?

Susanne Liffner | Isabelle Pehrson | Laura García-Calvo | Elizabeth Nedstrand | Stefan Zalavary | Mats Hammar | Heriberto Rodríguez-Martínez | Manuel Álvarez-Rodríguez

Department of Clinical and Experimental Medicine (IKE), Obstetrics and Gynaecology, Linköping University, Linköping, Sweden

Correspondence
Manuel Álvarez-Rodríguez, Department of Clinical and Experimental Medicine (IKE), Obstetrics and Gynaecology, Linköping University, Linköping, SE-58183, Sweden.
Email: manuel.alvarez-rodriguez@liu.se

Funding information
Forskningsrådet i Sydöstra Sverige, Grant/Award Number: 473121 and 745971; Lions Forskningsfond, Grant/Award Number: DNR LIU-2016-00641; Svenska Forskningsrådet Formas, Grant/Award Number: 2017-00946; ALF-Research (Region Östergötland), Grant/Award Number: LIO-698951

Abstract

Men affected with idiopathic infertility often display basic spermiogramme values similar to fertile individuals, questioning the diagnostic impact of the World Health Organization (WHO) thresholds used. This study explored sperm DNA fragmentation in single ejaculates from 14 fertile donors and 42 patients with idiopathic infertility providing semen for assisted reproductive techniques in a university fertility clinic. Each ejaculate was simultaneously studied for sperm DNA fragmentation by the flow cytometer-based sperm chromatin structure analysis (SCSA) and the new light-microscopy-based sperm chromatin dispersion assay (SCD-HaloSpermG2®), before and after sperm selection for in vitro fertilisation with a colloid discontinuous gradient. The WHO semen variables did not differ between groups, but DNA fragmentation after SCSA (DFI) or SCD (SDF) was significantly ($p < 0.05$) higher in patients (DFI: 40.2% ± 3.0 vs. SDF: 40.3% ± 1.4) than in fertile donors (DFI: 17.1% ± 2.1 vs. SDF: 20.9% ± 2.5). Sperm selection led to lower proportions of DNA-fragmented spermatozoa (DFI: 11.9 ± 1.7 vs. SCD: 10.0 ± 0.9, $p < 0.05$). The techniques output correlated highly and significantly ($r^2 = 0.82$). DNA fragmentation is confirmed as a relevant variable for scrutinising patients with idiopathic infertility, beyond the evidently insufficient WHO semen analyses. Since both techniques yielded similar results, the reduced necessity of complex equipment when running SCD ought to be considered for a clinical setting.

KEYWORDS
idiopathic infertility, SCD, SCSA, semen analyses, sperm DNA fragmentation

1 | INTRODUCTION

Involuntary childlessness affects at present ~90 million couples worldwide, that is one out of seven couples (one out of four in Western countries) ought to seek diagnosis and eventual alleviating treatment via assisted reproductive techniques (ART; Boivin, Bunting, Collins, & Nygren, 2007; Inhorn & Patrizio, 2015). Human infertility is a complex condition, caused by anomalies or dysfunctions of either one of the partners, couple-related or idiopathic (unknown aetiology; World Health Organization (WHO), 2010). Women age is a major risk factor (Fritz & Jindal, 2018; Shirasuna & Iwata, 2017), with fertility even after in vitro fertilisation (IVF) dropping

below 30% already by 35 years of age (Q-IVF, 2018). Male subfertility at similar age intervals is thought to be predisposed/caused by several factors all leading to low sperm count, sperm dysfunction or both (Levine et al., 2017; Mazur & Lipshultz, 2018).

Human ejaculates collected for diagnostics and preparation for ART are usually only assessed on the basis of conventional seminal parameters (WHO, 2010). These analyses, based on measurements of sperm concentration, motility and morphology, are difficult to standardise among different laboratories (Pacey, 2010), and thus, thresholds have been agreed by WHO (2010). Unfortunately, many men with results within "acceptable limits" remain diagnosed as idiopathically infertile even when using ART, that is no clear cause of the infertility has been found in either the women or the man (Pan, Hockenberry, Kirby, & Lipshultz, 2018). This indicates that sperm dysfunction is not fully diagnosed by the WHO-recommended methods, which are unable to determine neither the potential fertilising capacity nor provide prognosis of the (in)-fertility potential of the couple (Agarwal, Cho, & Esteves, 2016a; Lewis et al., 2013).

Use of IVF and the increasing use of forced fertilisation via intracytoplasmic sperm injection (ICSI) have undoubtedly ameliorated the condition of childlessness. The procedures are, however, symptomatic and do not provide diagnosis or specific treatment for the underlying pathologies. Moreover, their effectiveness has remained unchanged over the past 15 years, with ~30% of treatments resulting in birth (Q-IVF, 2018). Such stagnation of the results requires increasing our knowledge on the handling of semen, since both ARTs differ dramatically from the process of natural conception. This is particularly true regarding use of non-ejaculated spermatozoa, ICSI after testicular sperm extraction (elongated spermatids) or the unnatural mixing of ejaculate fractions and later removal of the seminal plasma (SP).

A damaged sperm chromatin, including a fragmented DNA, may impair the capability of the spermatozoa to fertilise. The first human clinical study showing a high correlation between sperm DNA fragmentation and pregnancy outcomes was done in an in vivo fertility study on 402 semen samples from 165 presumably fertile couples over 12 menstrual cycles (Evenson et al., 1999). The SCSA data from the male partners of 73 couples (group 1) achieving pregnancy during months 1–3 differed significantly from those of 40 couples (group 3) achieving pregnancy in months 4–12 ($p < 0.01$) and those of male partners of 32 couples (group 5) not achieving pregnancy ($p < 0.001$). Group 2 contained couples who had a miscarriage. Based on logistic regression, the % DFI was the best predictor for whether a couple would not achieve a pregnancy. Some 84% of males in group 1 had % DFI <15%, while no couples became pregnant in group 1 with >30%. It has also been shown that DNA fragmentation decreases the success of IVF (Wdowiak, Bakalchuk, & Bakalchuk, 2015) or ICSI (Avendaño, Franchi, Duran, & Oehninger, 2010), causes pregnancy loss (Brahem et al., 2011; Carlini et al., 2017; Osman, Alsomait, Seshadri, El-Toukhy, & Khalaf, 2015) and even reduces the health of the offspring (Jin et al., 2015; Coughlan et al., 2015; Simon et al., 2013; Virro, Larson-Cook, & Evenson, 2004). DNA fragmentation causes these effects often in relation to oocyte quality, suggesting a greater impact when ART is applied (Meseguer et al., 2011). Determination of DNA fragmentation in spermatozoa may thus be a relevant, complementary sperm parameter to reinforce current sperm analyses (Agarwal, Majzoub, et al., 2016b; Evgeni, Charalabopoulos, & Asimakopoulos, 2014; Giwercman et al., 2010; Kim, 2018; Lewis, 2015; Malić Vončina et al., 2016); particularly when ART is used (Bounartzi et al., 2016; Bungum et al., 2007; Yilmaz et al., 2010). Most interesting is the determination of sperm DNA fragmentation in cases of men diagnosed with idiopathic infertility, where semen variables are within "normal" limits (Bareh et al., 2016; Oleszczuk, Augustinsson, Bayat, Giwercman, & Bungum, 2013; Santi, Spaggiari, & Simoni, 2018).

Sperm DNA fragmentation has been evaluated with different techniques (Chohan, Griffin, Lafromboise, Jonge, & Carrell, 2006; Kim, 2018; Panner Selvam & Agarwal, 2018). Among these, the most popular for screening purposes is the sperm chromatin structure assay (SCSA), a 30-year-old flow cytometry/specific software-linked methodology with high repeatability and low variation (1%–3%; Evenson, 2017), but requires specialised technicians for running it. Another technique with increasing popularity is the commercially available new kit for the sperm chromatin dispersion testing (SCD, HaloSpermG2®; Zeqiraj et al., 2018). The SCD has high reproducibility (<3%) and does not require complex instruments becoming simpler and quick to run (Fernández et al., 2003). For each technique, clinical thresholds for the acceptable proportion of spermatozoa with DNA fragmentation have been established, 30% for the SCSA (Evenson, 2017) and 26% for SCD (Wiweko & Utami, 2017).

The aim of the present study was to explore the status of DNA fragmentation in WHO normozoospermic fertile men (sperm donors) and male partners of couples diagnosed with idiopathic infertility, both providing semen for ART at a university fertility clinic. The SCSA and SCD (HaloSpermG2®) tests were compared on spermatozoa prior to and after a colloid gradient sperm selection.

2 | MATERIALS AND METHODS

2.1 | Ethical approval

Ethical permissions were approved by the regional Ethical Committee in Linköping (EPN-Linköping, Dnr 2010/398-31; Dnr 2013/103-31; Dnr 2013/344-32 and 2015/387-31), including detailed patient information for individual written consent.

2.2 | Reagents and media

All reagents were obtained from Sigma-Aldrich (Sweden), unless otherwise stated. HaloSpermG2® was pursued from Hallotech DNA S.L. (Madrid, Spain).

2.3 | Study design and patient population

This is a case series study where the population under study was a cohort of semen donors (14, control) or patients ($n = 42$) undergoing

infertility investigation and/or ART treatment (IVF) at the referral Reproduction Medicine Centre, Linköping University Hospital, Linköping, Sweden, between October 2016 and October 2017. All subjects gave written informed consent for participation. The semen donors were fertile individuals (mean age 39.7 years), normozoospermic and with documented fertility. The patients (mean age 33.2 years) were partners in an infertile couple who, after more than 1 year of unprotected intercourse not leading to pregnancy, were andrologically investigated, classified as affected by idiopathic infertility, and were pending ART by IVF/ICSI. The diagnosis of idiopathic infertility (unexplained infertility) was based on the following criteria: one year of unprotected intercourse without pregnancy, unremarkable andrological story (no cryptorchidism, no genetic abnormalities, no cancer treatment (radiation or chemotherapy), no drug/alcohol/medicine abuse, high (>30) body mass index or other iatrogenic factors), normal volume (>1.5 ml), sperm concentration (>15 × 10^6 spermatozoa/ml) or total sperm number (>39 × 10^6 spermatozoa), sperm motility (≥32% progressively motile spermatozoa), >4% of morphologically normal spermatozoa (WHO, 2010) and absence of leukospermia. Moreover, absence of female factors in the partner (anovulation, tubal factor, endometriosis) was considered.

2.4 | Semen samples

Ejaculates (as bulk) were collected by masturbation after a recommended 2- to 5-day abstinence period (WHO, 2010), either at the clinic or at home (when deliverable within 120 min of ejaculation). All ejaculates, one per individual, were coded. Following routine assessment of sperm numbers and motility (see below), a 50-µl semen aliquot was centrifuged (10,000 *g* at 5°C for 10 min) and the resulting sperm pellet extended in TNE buffer (Trizma hydrochloride [10 mM], NaCl [150 mM] and EDTA [1 mM]) to 2 × 10^6 spermatozoa/ml and frozen at −80°C until analysed for DNA fragmentation. Two millilitre of the rest of the ejaculate was subjected to a two-step discontinuous colloid gradient using 40% and 80% PureSperm® (Nidacon International AB, Gothenburg, Sweden) following the manufacturer set-up. After centrifugation at 300 *g* at rt for 30 min, the sperm pellet at the bottom of the tube was retrieved, extended in maintaining media (G-IVF Plus, Vitrolife, Göteborg, Sweden) to be thereafter handled as above (centrifuged 10,000 *g*-TNE-80°C freezing) until analysis.

2.5 | Sperm motility

Sperm concentration (×10^6 sperm/ml), motility (%) and velocity (µm/s) and proportions of progressive sperm motility were assessed using an upright Zeiss Axio Scope A1 light microscope equipped with a 10 × phase contrast objective (Carl Zeiss, Stockholm, Sweden) connected via a CMOS camera (UEye, IDS Imaging Development Systems GmbH, Obersulm, Germany) to a computer holding the Qualisperm™ sperm analysis software (Biophos SA, Lausanne, Switzerland; www.biophos.com). Semen droplets (~24 × 10^4 sperm in 10 µl) were placed on a pre-warmed Menzel-Gläser pre-cleaned microscope slide (size: 76 × 26 mm; ThermoFisher Scientific, Waltham, MA, USA) covered by a pre-warmed coverslip (size: 18 × 18 mm; VWR, Stockholm, Sweden), on a thermal plate (Temp Controller 2000-2, Pecon GmbH, Erbach, Germany) kept at 38°C.

2.6 | DNA fragmentation analyses

Sperm DNA fragmentation was analysed using two different techniques (SCSA and HaloSpermG2®), following thawing in a water bath (37°C). Samples were kept thereafter on an ice bed until analysis. Each measurement was replicated twice for each method.

2.6.1 | Sperm chromatin structure analysis (SCSA)

Working solutions

TNE: Tris-HCl 10 mM, NaCl 150 mM and EDTA 1 mM; pH 7.4.

Acid detergent (AC): NaCl 150 mM, Triton X-100 0.1% v/v and HCl 80 mM.

Acridine orange (AO) working solution: Citric acid 33.4 mM, Na_2HPO_4 132.3 mM, NaCl 150 mM, EDTA 1mM, AO 6 mg/L; pH 6.

Procedure

The SCSA is based on the phenomenon that a 30-s treatment with pH 1.2 buffer denatures the DNA at the sites of single- or double-strand breaks, whereas normal double-stranded DNA remains intact. The spermatozoa are thereafter stained with the fluorescent DNA dye acridine orange, which differentially stains double- and single-stranded DNA. Sperm chromatin damage is quantified using flow cytometry measurements of the metachromatic shift of AO after blue light excitation, where the intact (double-stranded) DNA emits green fluorescence while the denatured (single-stranded) DNA emits red fluorescence. This shift is captured as fluorescence intensity cytogram patterns. The extent of DNA denaturation is expressed as DNA fragmentation index (DFI), which is the ratio of red (level of denatured DNA) to total fluorescence intensity (the total DNA). The SCSA procedure protocol followed the description of Evenson (Evenson, 2013; Evenson, Larson, & Jost, 2002). In brief, 400 µl of AC solution was added to 200 µl of standard sample (mixed samples with high heterogeneity) in a pre-cooled cytometer tube. After exactly 30 s, 1.2 ml of AO staining was added. The FL1-H (500 ± 5) and FL3-H (130 ± 5) were adjusted to thereafter proceed with all the donor/patient samples to be analysed at one time, without modifying the setting established after the standard sample. Sperm suspensions were analysed in duplicate in a Gallios™ flow cytometer (Beckman Coulter, Bromma, Sweden) equipped with standard optics: a violet laser (405 nm) with two colours, argon laser (488 nm) with five colours, and a HeNe laser (633 nm) with three colours. The filter configuration was as follows: Blue: FL1 550SP 525BP and FL3 655SP 620/30. The instrument was controlled via the Navios software (Beckman Coulter, Bromma, Sweden). Analyses of acquired data were performed using the Kaluza software (Beckman Coulter, Bromma, Sweden) on a separate PC. In all cases, 5,000 events were assessed per sample, with a flow rate of 200–250 cells/s. Further

calculations of the raw.fcs data in R-enviroment (package rflowcyt—Bioconductor) were done to obtain both the ratio of red/red + green fluorescence, where red is broken DNA and green is native DNA, as % DFI (DFI above 24.5%; DFI), and the measure of the proportion of immature spermatozoa having defects in the histone-to-protamine transition, normally occurring during sperm maturation in the epididymis, as % of HDS (High DNA Stainability; dependent of the green fluorescence; HDS).

2.6.2 | Sperm chromatin dispersion assay (SCD, HaloSpermG2®)

Sperm DNA fragmentation was also examined with the commercial kit HaloSpermG2®. This method is based on the characteristic halo formed when nuclear proteins are removed/uncoiled by acid denaturation of the spermatozoa. Sperm nuclei with severe DNA fragmentation form either a very small halo or no halo at all (i.e., no dispersion of DNA loops), while spermatozoa with little or without DNA fragmentation form a large halo (i.e., the DNA loops largely disperse). Following the manufacturer protocol, 50 µl of the thawed sperm sample was placed in a tube containing 100 µl of agarose (melted at 90°C and subsequently placed for five minutes at 37°C). A drop of 8 µl was deposited onto a super-coated glass slide, covered with a coverslip (avoiding bubble formation), and the slide/s placed on a 4°C cold surface for 5 min to solidify the agarose with the spermatozoa embedded within. The coverslip was gently removed, and the glass slide kept horizontally to allow application of the denaturant agent for 7 min at room temperature. After that, the denaturant was carefully removed (without shaking) to apply the lysis solution for 20 min. The slide was washed with abundant distilled water for 5 min and then dehydrated with increasing concentrations of ethanol (70% and 100%) each for 2 min. The drops were then stained with eosine for 7 min followed by thiazine staining for seven additional minutes. Finally, the excess of staining solutions was removed and the slides allowed to dry at room temperature. Visualisation was performed in an inverted bright field microscope (LEICA D100, Stockholm, Sweden). A minimum of 200 spermatozoa per sample were scored at ×400 magnification. In order to reduce bias, two persons counted the same sample. Calculation of the percentage of spermatozoa with fragmented DNA (as SDF) was performed dividing the number of fragmented + degraded spermatozoa by the total number of spermatozoa counted (×100). A positive control was run skipping the addition of the denaturant agent, where all spermatozoa were shown with halo. A negative control was run, where the lysis solution was skipped: all spermatozoa lacked halo.

2.7 | Statistical analysis

Statistical analysis, including calculation of coefficient of variation (CV; Standard deviation/mean × 100) and confidence interval (CI) at 95% confidence level, was performed in R-package using linear mixed-effects models. Residuals were tested for normality (Shapiro–Wilk test). The data of progression tests were normally distributed. Percentage data were arcsin square-root transformed when necessary. Individual samples were used as the grouping factor in the random part of the models. Results are shown as means ± standard deviation, unless otherwise stated. The Bland–Altman procedure was performed in order to analyse the agreement between the two different assays carried out. Pearson correlation (two-sided) was performed between different measurement points and different analysed parameters. A significant statistical level of $p < 0.05$ was used.

3 | RESULTS

The conventional WHO semen parameters did not statistically differ between the idiopathic infertile patients and fertile donors. The CV between the replicate analyses was 3.6% for the SCSA and 4.7% for the HaloSpermG2. The proportions of spermatozoa with DNA fragmentation in fertile donors and patients were similarly measured by either technique. For fertile semen donors, the sperm chromatin dispersion (SDF) was 20.9% ± 9.4 (range: 6.8–41.0; CV = 44.9%; 95% CI (CI = 15.976, 25.824) and the DNA fragmentation after SCSA (DFI) was 17.1% ± 7.8 (range: 6.9–31.2; CV = 45.6%; CI = 13.014, 21.186). The patients depicted an SDF of 40.1% ± 9.0 (range: 24.6–59.6; CV = 22.4%; CI = 37.378, 42.822) versus a DFI of 40.2% ± 19.3 (range: 2.3–80; CV = 48.0%; CI [34.363, 46.037). Values did not differ statistically within group (ns), but the fertile semen donors had significantly ($p < 0.05$) fewer spermatozoa with fragmented DNA measured with either method than patients with a diagnosis of idiopathic infertility. After the spermatozoa were selected by colloid discontinuous gradient in patients, their sperm motility increased to 89.7 ± 14.0% and sperm velocity to 43.4 ± 11.9 µm/s (Mean ± *SD*) and those depicting fragmented DNA were significantly fewer ($p < 0.05$; SDF: 10.0% ± 5.9 [2.3/30.9]) and (DFI: 11.9 ± 11.2 [0.6/53.5]) compared to the initial ejaculate, posing a tendency ($p > 0.05$) to reach fertile donor values.

Lower values of "High DNA Stainability" (HDS), a measure (%) of the proportion of immature spermatozoa having defects in the histone-to-protamine transition, were found in samples from patients after the colloid discontinuous gradient (6.7% ± 4.6 [2.4/22]) compared to the initial ejaculates, either from patients (13.2% ± 10.3 [1.6/42.2]) or from fertile donors (14.1% ± 10.2 [5.9/39.8]).

Both the SDF parameter yielded by the SCD assay (HaloSpermG2®) and that of the SCSA assay (DFI) were positively and highly ($R = 0.82$, $p < 0.05$) correlated in semen donors as well as patients. These results were confirmed through analysis of a Bland–Altman plot (Figure 1).

4 | DISCUSSION

The present study confirmed the value of both SCSA and SCD (HaloSpermG2®) to determine sperm DNA fragmentation in semen with apparently similar WHO standard semen parameters. Men

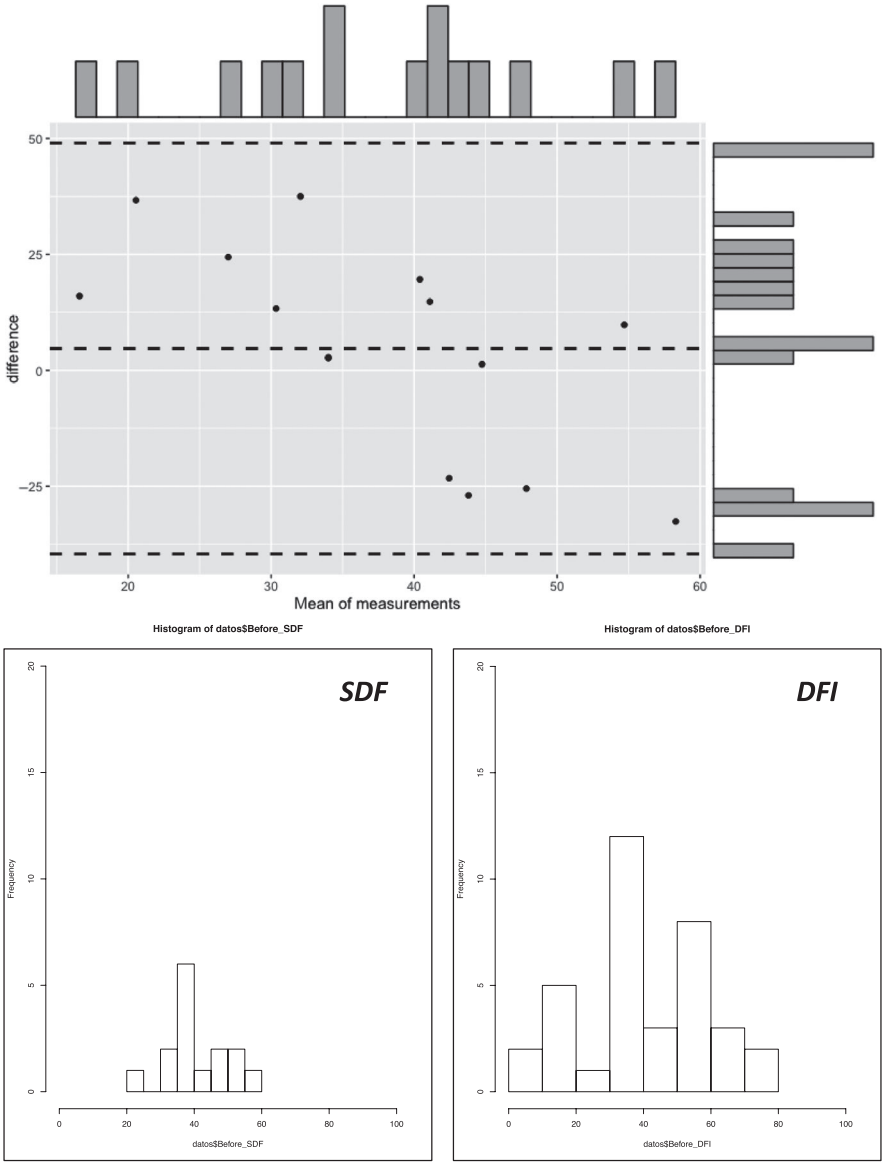

FIGURE 1 Bland–Altman plots to illustrate that both analytical techniques, sperm chromatin dispersion test HalospermG2 (SCD, as SDF) versus sperm chromatin structure assay (SCSA, as DFI), were equally effective in measuring sperm DNA fragmentation in semen from patients, partners of couples diagnosed with idiopathic infertility

with these conventional semen values ought to be considered normozoospermic, despite being either derived from fertility-proven semen donors or from male partners of couples diagnosed with unexplained infertility. Irrespective of the technique used, the infertile patients showed significantly higher DNA fragmentation rates compared to fertile donors, well beyond values considered clinically normal thresholds (Evenson, 2017; Wiweko & Utami, 2017). To this point, DNA fragmentation adds a diagnostic value for the screening of males diagnosed with idiopathic infertility, beyond what is praxis during andrological routine evaluation.

Of major interest were the results of the correlations between the two techniques, providing at hand that they were basically similar in disclosing DNA fragmentation values in most paired samples. The SCSA is today the most commonly used testing method for DNA fragmentation in clinical evaluations, measuring only single-stranded DNA fragments. The method is based on the higher vulnerability of DNA from damaged spermatozoa to acid detergent denaturation, which enhances the binding of acridine orange as an aggregate to the single-stranded DNA differentiating it from the intact double-stranded DNA (Evenson et al., 2002). The parameter most commonly followed is the DFI (DFI, %) which indicates the relative number of spermatozoa with DNA damage. Moreover, the SCSA provides a measure (%) of the proportion of immature spermatozoa having defects in the histone-to-protamine transition, which normally occurs during sperm maturation in the epididymis, under the acronym HDS. Following both parameters, a clinical prognosis for fertility after ART has been established, where a combination of DFI higher than 30% and HDS higher than 15% would lead to fertilisation failure, low blastocyst development or no pregnancy (Evenson, 2017; Virro et al., 2004). The SCSA analyses large numbers of spermatozoa under short time, and it has a high reproducibility (1%–3% intra-assay variation [Evenson, 2017]), becoming statistically robust, proven by a long-lasting use worldwide. However, it has disadvantages for application in a clinical setting, since it requires technical know-how, handling specific software and an expensive flow cytometry equipment.

The SCD assay has also been used for a long time (Fernández et al., 2003), being further developed under time to attempt becoming an economical and management alternative to SCSA (Feijó & Esteves, 2014). As such, it has been adopted by many IVF clinics since it is simple, quick, does not require expensive or complicated instruments and, moreover, has high reproducibility (<3% intra-assay variation; Panner Selvam & Agarwal, 2018; Zeqiraj et al., 2018). The CV for the reference sample and replicates assayed in the present study were well within these values, confirming previous studies (Erenpreiss, Bungum, et al., 2006a). As for SCSA, the new SCD test HaloSpermG2® also involves acid denaturation, which generates single-stranded DNA motifs from DNA breaks. This acid detergent treatment causes uncoiling of nuclear proteins from the chromatin, allowing the highly coiled intact DNA to expand and to form a halo over the sperm head; the larger the halo, the less DNA damage present. The following lysis step in SCD-HaloSpermG2® causes deproteinisation of the chromatin which suppresses the formation of a halo. The mechanism for this halo suppression is unknown, but the suppression of halo formation is not observed in spermatozoa with unfragmented DNA. The major advantage of the method is the easiness to count spermatozoa with damaged DNA (no halo/small halo) from the rest (large halos). However, even considering these advantages, the periphery of the halo where low-density nucleoids are often located is faint and yields low-contrast images that can lead to errors since the halo border is difficult to distinguish. Moreover, sometimes halos are not all in the same focal plane, requiring the operator to be confident in managing the microscope during counting. Lastly, contaminant cells other than spermatozoa can also produce halo and they must be distinguished.

Several other methods can be used to determine DNA damage in spermatozoa (rev by Rodriguez-Martinez, 2014), such as the terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick-end labelling, the acridine orange test, the tritium-labelled 3H-actinomycin D incorporation assay, the in situ nick translation, the DNA breakage detection-fluorescence in situ hybridisations and the single-cell gel electrophoresis assay (COMET) including the variants of alkaline COMET and the neutral COMET, the latter with specific threshold values for prediction of male infertility (Ribas-Maynou et al., 2013). Relevant for the newly available and hereby used HaloSpermG2® is, moreover being less cumbersome than some of the above listed methods or SCSA, the inclusion of a reduced acid detergent treatment step (20 min) as well as a lysis step that reduces/eliminates false-positive counting.

In the present study, spermatozoa from patients diagnosed with idiopathic infertility showed, despite having a routine spermiogramme within normal WHO criteria limits, an increased DNA fragmentation level, measured by two analytical techniques, compared with fertile semen donors. Such increased levels would be one reason for a decreased fertility, where a dysfunction at the chromatin level would not be detected using conventional semen analyses. Fertile donors were all well under the threshold for in vivo fertility determined for the analytical techniques (DFI: 17.1% ± 8.0; SDF: 20.9% ± 9.4). The values did not differ statistically. Single ejaculates (one per person) were examined in the present study, the rationale being that the exams for DNA fragmentation were intended on semen that was aimed for IVF. The CV and 95% CIs for DFI or SDF were similarly large for fertile semen donors and infertile patients. The large CV would be caused by the use of single samples, considering that the variation among ejaculates within individual was reported large, irrespective of the number of ejaculates examined per person (Erenpreiss, Bungum, et al., 2006a).

The semen from the fertile donors used for IVF has led to live babies born (data not shown). This might reinforce the value of a low DNA fragmentation rate for fertility. Semen from the patients was also used for IVF, after a cleansing/selection procedure based on a commercial discontinuous colloid gradient. The spermatozoa selected by the procedure were also examined by SCSA and SCD, and levels of DNA fragmentation were significantly decreased by the selection procedure, basically threefold after the gradient. The proportion of spermatozoa after gradient selection with fragmented DNA even reached levels below those registered in the normozoospermic semen donors. A follow-up of the IVF-results for these patients revealed a lack of correlation between DNA fragmentation level after the gradient with the rates of fertilisation, blastocyst or live births, with pregnancies-to-term being established even with significantly higher DNA fragmentation values (not different neither for DFI nor SDF). Noteworthy, the levels of DNA fragmentation for successful pregnancies-to-term were always below the clinical threshold established for SCSA or SCD, for example, <24.5% (Mean ± *SD* [min/max]): No pregnancy-to-term (DFI: 9.6% ± 8.4 [0.6–34.4] versus SDF: 8.7% ± 6.0 [2.3–30.9]) versus pregnancy-to-term (DFI: 12.9% ± 5.1 [6.6–22.4] versus SDF: 17.3% ± 14.5 [1.4–53.4]). Such results should be considered in the light of the restricted number of individuals constituting the cohorts. Moreover, conflicting results have been presented in the literature regarding the relationship of DNA fragmentation measured by either technique and the various end-points measured: fertilisation, blastocyst rate, early pregnancy diagnosis, birth rate or the possible complications that sometimes occur (abortion, miscarriage, etc). Some studies praise the SCSA for their prognostic value (Evenson, 2017; Evenson et al., 2002) while others do the same for the SCD (Comhaire, Messiaen, & Decleer, 2018; Fernández, Cajigal, López-Fernández, & Gosálvez, 2011; Pregl Breznik, Kovačič, & Vlaisavljević, 2013; Zheng et al., 2018). Caution has been recommended when considering prognosis after IVF or ICSI using solely DNA fragmentation (Anifandis et al., 2015; Cissen et al., 2016; Erenpreiss, Spano, Erenpreisa, Bungum, & Giwercman, 2006b; Zhang et al., 2015) particularly when colloid gradients are used since other methods for sperm selection are more valuable, as swim-up (Zandieh et al., 2018).

The relevance of the present study is that both methods showed similar results, with a relevant statistical correlation, and that the diagnostics was valuable, since a possible explanation for the infertility among the participating patients could have been the high proportion of spermatozoa with damaged DNA (Oguz et al., 2018). When their semen was subjected to gradient selection, the proportion of damaged spermatozoa decreased and

pregnancies-to-term were established. However, it should be considered that gradient selection, although selecting for DNA intactness using the SCD-HaloSpermG2® assay, does not seem to statistically correlate with pregnancy-to-term after IVF (Comhaire et al., 2018; Zheng et al., 2018) or ICSI (Wang et al., 2014).

5 | CONCLUSION

In conclusion, SCSA and SCD-HaloSpermG2® are comparable techniques for measuring sperm DNA fragmentation, with a logistic advantage for the latter if intending application in a clinical setting. Further studies with larger cohorts are warranted to confirm these results.

ACKNOWLEDGEMENTS

Marie Rubér and Alejandro Vicente-Carrillo, Linköping University are warmly acknowledged for their kind assistance in the primary handling of ejaculates. The study was supported by grants from ALF-Research (Region Östergötland, LIO-698951), FORSS (Forskningsrådet i Sydöstra Sverige, Grant 473121 and Grant 745971), Lions Forskningsfond (DNR LIU-2016-00641) and the Swedish Research Council FORMAS (Grant 2017-00946), Stockholm, Sweden; including student grants for I.P. (Region Östergötland, LIO-70460/802571/833121) and L.G-C (Formas).

CONFLICT OF INTEREST

The authors declare no conflicts of interest to disclose.

AUTHORS' CONTRIBUTIONS

M.A.-R., S.L., M.H. and H.R.-M. designed the experiments. M.A.-R., I.P., L.G.-C. and S.L. executed the experiments. M.A.-R. performed analyses of data and wrote the first draft of the manuscript. E.N. recruited patients. S.Z. secured semen samples. All authors read, contributed and approved the final manuscript. M.A.-R. and H.R.-M. secured funding.

ORCID

Manuel Álvarez-Rodríguez https://orcid.org/0000-0003-0120-354X

REFERENCES

Agarwal, A., Cho, C. L., & Esteves, S. C. (2016a). Should we evaluate and treat sperm DNA fragmentation? *Current Opinion in Obstetrics and Gynecology*, *28*(3), 164–171. https://doi.org/10.1097/GCO.0000000000000271

Agarwal, A., Majzoub, A., Esteves, S. C., Ko, E., Ramasamy, R., & Zini, A. (2016b). Clinical utility of sperm DNA fragmentation testing: Practice recommendations based on clinical scenarios. *Translational Andrology and Urology*, *5*(6), 935–950. https://doi.org/10.21037/tau.2016.10.03

Anifandis, G., Bounartzi, T., Messini, C. I., Dafopoulos, K., Markandona, R., Sotiriou, S., … Messinis, I. E. (2015). Sperm DNA fragmentation measured by Halosperm does not impact on embryo quality and ongoing pregnancy rates in IVF/ICSI treatments. *Andrologia*, *47*(3), 295–302. https://doi.org/10.1111/and.12259

Avendaño, C., Franchi, A., Duran, H., & Oehninger, S. (2010). DNA fragmentation of normal spermatozoa negatively impacts embryo quality and intracytoplasmic sperm injection outcome. *Fertility and Sterility*, *94*(2), 549–557. https://doi.org/10.1016/j.fertnstert.2009.02.050

Bareh, G. M., Jacoby, E., Binkley, P., Chang, T.-C.-A., Schenken, R. S., & Robinson, R. D. (2016). Sperm deoxyribonucleic acid fragmentation assessment in normozoospermic male partners of couples with unexplained recurrent pregnancy loss: A prospective study. *Fertility and Sterility*, *105*(2), 329–336.e1. https://doi.org/10.1016/j.fertnstert.2015.10.033

Boivin, J., Bunting, L., Collins, J. A., & Nygren, K. G. (2007). International estimates of infertility prevalence and treatment-seeking: Potential need and demand for infertility medical care. *Human Reproduction*, *22*(6), 1506–1512. https://doi.org/10.1093/humrep/dem046

Bounartzi, T., Dafopoulos, K., Anifandis, G., Messini, C. I., Koutsonikou, C., Kouris, S., … Messinis, I. E. (2016). Pregnancy prediction by free sperm DNA and sperm DNA fragmentation in semen specimens of IVF/ICSI-ET patients. *Human Fertility (Cambridge)*, *19*(1), 56–62. https://doi.org/10.3109/14647273.2016.1157629

Brahem, S., Mehdi, M., Landolsi, H., Mougou, S., Elghezal, H., & Saad, A. (2011). Semen parameters and sperm DNA fragmentation as causes of recurrent pregnancy loss. *Urology*, *78*(4), 792–796. https://doi.org/10.1016/j.urology.2011.05.049

Bungum, M., Humaidan, P., Axmon, A., Spano, M., Bungum, L., Erenpreiss, J., & Giwercman, A. (2007). Sperm DNA integrity assessment in prediction of assisted reproduction technology outcome. *Human Reproduction*, *22*(1), 174–179. https://doi.org/10.1093/humrep/del326

Carlini, T., Paoli, D., Pelloni, M., Faja, F., Dal Lago, A., Lombardo, F., … Gandini, L. (2017). Sperm DNA fragmentation in Italian couples with recurrent pregnancy loss. *Reproductive Biomedicine Online*, *34*(1), 58–65. https://doi.org/10.1016/j.rbmo.2016.09.014

Chohan, K. R., Griffin, J. T., Lafromboise, M., De Jonge, C. J., & Carrell, D. T. (2006). Comparison of chromatin assays for DNA fragmentation evaluation in human sperm. *Journal of Andrology*, *27*(1), 53–59. https://doi.org/10.2164/jandrol.05068

Cissen, M., Wely, M. V., Scholten, I., Mansell, S., Bruin, J. P. D., Mol, B. W., … Hamer, G. (2016). Measuring sperm DNA fragmentation and clinical outcomes of medically assisted reproduction: A systematic review and meta-analysis. *PLoS ONE*, *11*(11), e0165125. https://doi.org/10.1371/journal.pone.0165125

Comhaire, F., Messiaen, A., & Decleer, W. (2018). A mathematical model predicting the individual outcome of IVF through sperm-analysis: The role of the HaloSpermG2® DNA fragmentation test. *Medical Hypotheses*, *117*, 50–53. https://doi.org/10.1016/j.mehy.2018.05.021

Erenpreiss, J., Bungum, M., Spano, M., Elzanaty, S., Orbidans, J., & Giwercman, A. (2006a). Intra-individual variation in sperm chromatin structure assay parameters in men from infertile couples: Clinical implications. *Human Reproduction*, *21*(8), 2061–2064. https://doi.org/10.1093/humrep/der328

Erenpreiss, J., Spano, M., Erenpreisa, J., Bungum, M., & Giwercman, A. (2006b). Sperm chromatin structure and male fertility: Biological and clinical aspects. *Asian Journal of Andrology*, *8*(1), 11–29. https://doi.org/10.1111/j.1745-7262.2006.00112.x

Evenson, D. P. (2013). Sperm chromatin structure assay (SCSA®). *Methods in Molecular Biology*, *927*, 147–164. https://doi.org/10.1007/978-1-62703-038-0_14

Evenson, D. P. (2017). Evaluation of sperm chromatin structure and DNA strand breaks is an important part of clinical male fertility assessment. *Translational Andrology and Urology*, *6*(S4), S495–S500. https://doi.org/10.21037/tau.2017.07.20

Evenson, D. P., Jost, L. K., Zinaman, M. J., Clegg, E., Purvis, K., deAngelis, P., & Clausen, O. P. (1999). Utility of the sperm chromatin structure assay (SCSA) as a diagnostic and prognostic tool in the human fertility clinic. *Human Reproduction*, *14*(4), 1039–1049. https://doi.org/10.1093/humrep/14.4.1039

Evenson, D. P., Larson, K. L., & Jost, L. K. (2002). Sperm chromatin structure assay: Its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. *Journal of Andrology*, *23*(1), 25–43. https://doi.org/10.1002/j.1939-4640.2002.tb02599.x

Evgeni, E., Charalabopoulos, K., & Asimakopoulos, B. (2014). Human sperm DNA fragmentation and its correlation with conventional semen parameters. *Journal of Reproduction and Infertility*, *15*(1), 2–14.

Feijó, C. M., & Esteves, S. C. (2014). Diagnostic accuracy of sperm chromatin dispersion test to evaluate sperm deoxyribonucleic acid damage in men with unexplained infertility. *Fertility and Sterility*, *101*(1), 58–63.e3. https://doi.org/10.1016/j.fertnstert.2013.09.002

Fernández, J. L., Cajigal, D., López-Fernández, C., & Gosálvez, J. (2011). Assessing sperm DNA fragmentation with the sperm chromatin dispersion test. *Methods in Molecular Biology*, *682*, 291–301. https://doi.org/10.1007/978-1-60327-409-8_21

Fernández, J. L., Muriel, L., Rivero, M. T., Goyanes, V., Vazquez, R., & Alvarez, J. G. (2003). The sperm chromatin dispersion test: A simple method for the determination of sperm DNA fragmentation. *Journal of Andrology*, *24*(1), 59–66. https://doi.org/10.1002/j.1939-4640.2003.tb02641.x

Fritz, R., & Jindal, S. (2018). Reproductive aging and elective fertility preservation. *Journal of Ovarian Research*, *11*(1), 66. https://doi.org/10.1186/s13048-018-0438-4

Giwercman, A., Lindstedt, L., Larsson, M., Bungum, M., Spano, M., Levine, R. J., & Rylander, L. (2010). Sperm chromatin structure assay as an independent predictor of fertility in vivo: a case-control study. *International Journal of Andrology*, *33*(1), e221–e227. https://doi.org/10.1111/j.1365-2605.2009.00995.x

Inhorn, M. C., & Patrizio, P. (2015). Infertility around the globe: New thinking on gender, reproductive technologies and global movements in the 21st century. *Human Reproduction Update*, *21*(4), 411–426. https://doi.org/10.1093/humupd/dmv016

Jin, J., Pan, C., Fei, Q., Ni, W., Yang, X., Zhang, L., & Huang, X. (2015). Effect of sperm DNA fragmentation on the clinical outcomes for in vitro fertilization and intracytoplasmic sperm injection in women with different ovarian reserves. *Fertility and Sterility*, *103*(4), 910–916. https://doi.org/10.1016/j.fertnstert.2015.01.014

Kim, G. Y. (2018). What should be done for men with sperm DNA fragmentation? *Clinical and Experimental Reproductive Medicine*, *45*(3), 101–109. https://doi.org/10.5653/cerm.2018.45.3.101

Levine, H., Jørgensen, N., Martino-Andrade, A., Mendiola, J., Weksler-Derri, D., Mindlis, I., … Swan, S. H. (2017). Temporal trends in sperm count: A systematic review and meta-regression analysis. *Human Reproduction Update*, *23*(6), 646–659. https://doi.org/10.1093/humupd/dmx022

Lewis, S. E. M. (2015). Should sperm DNA fragmentation testing be included in the male infertility work-up? *Reproductive Biomedicine Online*, *31*(2), 134–137. https://doi.org/10.1016/j.rbmo.2015.05.006

Lewis, S. E. M., John Aitken, R., Conner, S. J., Iuliis, G. D., Evenson, D. P., Henkel, R., … Gharagozloo, P. (2013). The impact of sperm DNA damage in assisted conception and beyond: Recent advances in diagnosis and treatment. *Reproductive Biomedicine Online*, *27*(4), 325–337. https://doi.org/10.1016/j.rbmo.2013.06.014

Malić Vončina, S., Golob, B., Ihan, A., Kopitar, A. N., Kolbezen, M., & Zorn, B. (2016). Sperm DNA fragmentation and mitochondrial membrane potential combined are better for predicting natural conception than standard sperm parameters. *Fertility and Sterility*, *105*(3), 637–644.e1. https://doi.org/10.1016/j.fertnstert.2015.11.037

Mazur, D. J., & Lipshultz, L. I. (2018). Infertility in the aging male. *Current Urology Reports*, *19*(7), 54. https://doi.org/10.1007/s11934-018-0802-3

Meseguer, M., Santiso, R., Garrido, N., García-Herrero, S., Remohí, J., & Fernandez, J. L. (2011). Effect of sperm DNA fragmentation on pregnancy outcome depends on oocyte quality. *Fertility and Sterility*, *95*(1), 124–128. https://doi.org/10.1016/j.fertnstert.2010.05.055

Oguz, Y., Guler, I., Erdem, A., Mutlu, M. F., Gumuslu, S., Oktem, M., … Erdem, M. (2018). The effect of swim-up and gradient sperm preparation techniques on deoxyribonucleic acid (DNA) fragmentation in subfertile patients. *Journal of Assisted Reproduction and Genetics*, *35*(6), 1083–1089. https://doi.org/10.1007/s10815-018-1163-z

Oleszczuk, K., Augustinsson, L., Bayat, N., Giwercman, A., & Bungum, M. (2013). Prevalence of high DNA fragmentation index in male partners of unexplained infertile couples. *Andrology*, *1*(3), 357–360. https://doi.org/10.1111/j.2047-2927.2012.00041.x

Osman, A., Alsomait, H., Seshadri, S., El-Toukhy, T., & Khalaf, Y. (2015). The effect of sperm DNA fragmentation on live birth rate after IVF or ICSI: A systematic review and meta-analysis. *Reproductive Biomedicine Online*, *30*(2), 120–127. https://doi.org/10.1016/j.rbmo.2014.10.018

Pacey, A. A. (2010). Quality assurance and quality control in the laboratory andrology. *Asian Journal of Andrology*, *12*(1), 21–25. https://doi.org/10.1038/aja.2009.16

Pacey, A. A., Coughlan, C., Clarke, H., Cutting, R., Saxton, J., Waite, S., … Li, T. (2015). Sperm DNA fragmentation, recurrent implantation failure and recurrent miscarriage. *Asian Journal of Andrology*, *17*(4), 681–685. https://doi.org/10.4103/1008-682X.144946

Pan, M. M., Hockenberry, M. S., Kirby, E. W., & Lipshultz, L. I. (2018). Male infertility diagnosis and treatment in the era of in vitro fertilization and intracytoplasmic sperm injection. *Medical Clinics of North America*, *102*(2), 337–347. https://doi.org/10.1016/j.mcna.2017.10.008

Panner Selvam, M. K., & Agarwal, A. (2018). A systematic review on sperm DNA fragmentation in male factor infertility: Laboratory assessment. *Arab Journal of Urology*, *16*(1), 65–76. https://doi.org/10.1016/j.aju.2017.12.001

Pregl Breznik, B., Kovačič, B., & Vlaisavljević, V. (2013). Are sperm DNA fragmentation, hyperactivation, and hyaluronan-binding ability predictive for fertilization and embryo development in in vitro fertilization and intracytoplasmic sperm injection? *Fertility and Sterility*, *99*(5), 1233–1241. https://doi.org/10.1016/j.fertnstert.2012.11.048

Q-IVF. (2018). http://www.ucr.uu.se/qivf (last access 2019-01-30).

Ribas-Maynou, J., García-Peiró, A., Fernández-Encinas, A., Abad, C., Amengual, M. J., Prada, E., … Benet, J. (2013). Comprehensive analysis of sperm DNA fragmentation by five different assays: TUNEL assay, SCSA, SCD test and alkaline and neutral Comet assay. *Andrology*, *1*(5), 715–722. https://doi.org/10.1111/j.2047-2927.2013.00111.x

Rodriguez-Martinez, H. (2014). Semen evaluation and handling: emerging techniques and future development. In P. J. Chenoweth, & S. Lorton (Eds.), *Animal andrology: theory and applications*, Ch. 19 (pp. 509–549). Oxfordshire, UK: Cabi. (ISBN-10: 1780643160).

Santi, D., Spaggiari, G., & Simoni, M. (2018). Sperm DNA fragmentation index as a promising predictive tool for male infertility diagnosis and treatment management –meta-analyses. *Reproductive Biomedicine Online*, *37*(3), 315–326. https://doi.org/10.1016/j.rbmo.2018.06.023

Shirasuna, K., & Iwata, H. (2017). Effect of aging on the female reproductive function. *Contraception and Reproductive Medicine*, *3*, 2–23. https://doi.org/10.1186/s40834-017-0050-9

Simon, L., Proutski, I., Stevenson, M., Jennings, D., McManus, J., Lutton, D., & Lewis, S. E. (2013). Sperm DNA damage has a negative association with live-birth rates after IVF. *Reproductive Biomedicine Online*, *26*(1), 68–78. https://doi.org/10.1016/j.rbmo.2012.09.019

Virro, M. R., Larson-Cook, K. L., & Evenson, D. P. (2004). Sperm chromatin structure assay (SCSA) parameters are related to fertilization, blastocyst development, and ongoing pregnancy in in vitro fertilization and intracytoplasmic sperm injection cycles. *Fertility and Sterility*, *81*(5), 1289–1295. https://doi.org/10.1016/j.fertnstert.2003.09.063

Wang, M., Sun, J., Wang, L. I., Gao, X., Lu, X., Wu, Z., … Wu, Y. U. (2014). Assessment of density gradient centrifugation (DGC) and sperm chromatin dispersion (SCD) measurements in couples with male factor infertility undergoing ICSI. *Journal of Assisted Reproduction and Genetics*, *31*(12), 1655–1663. https://doi.org/10.1007/s10815-014-0339-4

Wdowiak, A., Bakalchuk, S., & Bakalchuk, G. (2015). The effect of sperm DNA fragmentation on the dynamics of the embryonic development in intracytoplasmatic sperm injection. *Reproductive Biology*, *15*(2), 94–100. https://doi.org/10.1016/j.repbio.2015.03.003

Wiweko, B., & Utami, P. (2017). Predictive value of sperm deoxyribonucleic acid (DNA) fragmentation index in male infertility. *Basic and Clinical Andrology*, *27*, 1. https://doi.org/10.1186/s12610-016-0046-3

World Health Organization (WHO). (2010). *Department of reproductive health and research. Fifth edition WHO laboratory manual for the examination and processing of human semen*.

Yilmaz, S., Zergeroglu, A. D., Yilmaz, E., Sofuoglu, K., Delikara, N., & Kutlu, P. (2010). Effects of sperm DNA fragmentation on semen parameters and ICSI outcome determined by an improved SCD test Halosperm. *International Journal of Fertility and Sterility*, *4*, 73–78.

Zandieh, Z., Vatannejad, A., Doosti, M., Zabihzadeh, S., Haddadi, M., Bajelan, L., … Amanpour, S. (2018). Comparing reactive oxygen species and DNA fragmentation in semen samples of unexplained infertile and healthy fertile men. *Irish Journal of Medical Science*, *187*(3), 657–662. https://doi.org/10.1007/s11845-017-1708-7

Zeqiraj, A., Beadini, S., Beadini, N., Aliu, H., Gashi, Z., Elezaj, S., … Shabani, A. (2018). Male infertility and sperm DNA fragmentation. *Open Access Macedonian Journal of Medical Sciences*, *6*(8), 1342–1345. https://doi.org/10.3889/oamjms.2018.311

Zhang, Z., Zhu, L., Jiang, H., Chen, H., Chen, Y., & Dai, Y. (2015). Sperm DNA fragmentation index and pregnancy outcome after IVF or ICSI: A meta-analysis. *Journal of Assisted Reproduction and Genetics*, *32*(1), 17–26. https://doi.org/10.1007/s10815-014-0374-1

Zheng, W.-W., Song, G. E., Wang, Q.-L., Liu, S.-W., Zhu, X.-L., Deng, S.-M., … Tan, Y. (2018). Sperm DNA damage has a negative effect on early embryonic development following in vitro fertilization. *Asian Journal of Andrology*, *20*(1), 75–79. https://doi.org/10.4103/aja.aja_19_17

How to cite this article: Liffner S, Pehrson I, García-Calvo L, et al. Diagnostics of DNA fragmentation in human spermatozoa: Are sperm chromatin structure analysis and sperm chromatin dispersion tests (SCD-HaloSpermG2®) comparable? *Andrologia*. 2019;51:e13316. https://doi.org/10.1111/and.13316