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

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Diagnostics of DNA fragmentation in human spermatozoa: are Sperm Chromatin Structure Analysis (SCSA) and Sperm Chromatin Dispersion tests (SCD-HaloSpermG2®) comparable?

Running Head: Sperm DNA fragmentation in idiopathic infertility

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Men affected with idiopathic infertility often display basic spermiogramme values similar to fertile individuals, questioning the diagnostic impact of the 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 (ART) in a University fertility clinic. Each ejaculate were 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 IVF 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: $40.2\%\pm 3.0$ vs. SDF: $40.3\%\pm 1.4$) than in fertile donors (DFI: $17.1\%\pm 2.1$ vs. SDF: $20.9\%\pm 2.5$). 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 scrutinizing 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.

Key Words: idiopathic infertility, semen analyses, sperm DNA fragmentation, SCSA, SCD.

1 Introduction

Involuntary childlessness affects at present ~90 million couples worldwide, i.e. 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 etiology) (World Health Organization [WHO], 2010). Women age is a major risk factor (Fritz & Jindal, 2018; Shirasuna & Iwata 2017), with fertility even after 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 assisted reproductive techniques (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 standardize 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, i.e. 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 fertilizing capacity nor provide prognosis of the (in)-fertility potential of the couple (Agarwal, Cho & Esteves, 2016a; Lewis *et al.*, 2013).

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

70

71 A damaged sperm chromatin, including a fragmented DNA, may impair the capability
72 of the spermatozoa to fertilize. The first human clinical study showing a high
73 correlation between sperm DNA fragmentation and pregnancy outcomes was done in
74 an *in vivo* fertility study on 402 semen samples from 165 presumably fertile couples
75 over 12 menstrual cycles (Evenson *et al.*, 1999). The SCSA data from the male partners
76 of 73 couples (group 1) achieving pregnancy during months 1-3 differed significantly
77 from those of 40 couples (group 3) achieving pregnancy in months 4-12 ($P < 0.01$) and
78 those of male partners of 32 couples (group 5) not achieving pregnancy ($P < 0.001$).
79 Group 2 contained couples who had a miscarriage. Based on logistic regression, the
80 %DFI was the best predictor for whether a couple would not achieve a pregnancy.
81 Some 84% of males in group 1 had %DFI $< 15\%$, while no couples became pregnant in
82 group 1 with $> 30\%$. It has also been shown that DNA fragmentation decrease the
83 success of IVF (Wdowiak, Bakalchuk & Bakalchuk, 2015) or ICSI (Avendaño,

Franchi, Duran & Oehninger, 2010), cause pregnancy loss (Brahem *et al.*, 2011; Carlini *et al.*, 2017; Osman, Alsomait, Seshadri, El-Toukhy & Khalaf, 2015), and even reduce the health of the offspring (Coughlan *et al.*, 2015; Jin *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, Esteves, Ko, Ramasamy & Zini, 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, De 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 specialized 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%), 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 purchased 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 (RMC), 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 one 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 ($> 15 \times 10^6$ spermatozoa/mL) or total sperm number ($>39 \times 10^6$ spermatozoa), sperm motility ($\geq 32\%$ 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).

2.4 Semen samples

Ejaculates (as bulk) were collected by masturbation after a recommended 2-5-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 μ L-semen aliquot was centrifuged ($10,000 \times g$ at 5 °C for 10 min) and the resulting sperm pellet extended in TNE-buffer (Trizma hydrochloride (10 mM), NaCl (150 mM), EDTA (1mM)) to 2×10^6 spermatozoa/mL and frozen at -80°C until analysed for DNA fragmentation. Two mL 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 xg 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 10,000xg-TNE-80°C freezing) until analysis.

2.5 Sperm motility

Sperm concentration ($\times 10^6$ sperm/mL), motility (%) and velocity ($\mu\text{m}/\text{sec}$) and proportions of progressive sperm motility were assessed using an upright Zeiss Axio Scope A1 light microscope equipped with a $10\times$ 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 QualispermTM sperm analysis software (Biophos SA, Lausanne, Switzerland; www.biophos.com). Semen droplets ($\sim 24 \times 10^4$ sperm in 10 μl) were placed on a pre-warmed Menzel-Gläser pre-cleaned microscope slide (size: 76 $\times$ 26 mm; ThermoFisher Scientific, Waltham, MA, USA) covered by a pre-warmed coverslip (size: 18 $\times$ 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.

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.

182 *Acridine orange (AO) working solution:* Citric acid 33.4 mM, Na₂HPO₄ 132.3 mM,
183 NaCl 150 mM, EDTA 1mM, AO 6 mg/L; pH 6.

184 ***Procedure***

185 The SCSA is based on the phenomenon that a 30 sec treatment with pH 1.2 buffer
186 denatures the DNA at the sites of single- or double-strand breaks, whereas normal
187 double-stranded DNA remains intact. The spermatozoa are thereafter stained with the
188 fluorescent DNA dye acridine orange, which differentially stains double- and single-
189 stranded DNA. Sperm chromatin damage is quantified using flow cytometry
190 measurements of the metachromatic shift of AO after blue light excitation, where the
191 intact (double-stranded) DNA emits green fluorescence while the denatured (single-
192 stranded) DNA emits red fluorescence. This shift is captured as fluorescence intensity
193 cytogram patterns. The extent of DNA denaturation is expressed as DNA
194 Fragmentation Index (DFI), which is the ratio of red (level of denatured DNA) to total
195 fluorescence intensity (the total DNA). The SCSA procedure protocol followed the
196 description of Evenson ([Evenson, Larson & Jost, 2002](#); [Evenson, 2013](#)). In brief, 400
197 µL of AC solution were added to 200 µL of standard sample (mixed samples with high
198 heterogeneity) in a pre-cooled cytometer tube. After exactly 30 sec, 1.2 mL of AO
199 staining were added. The FL1-H (500±5) and FL3-H (130±5) were adjusted to
200 thereafter proceed with all the donor/patient samples to be analysed at one time, without
201 modifying the setting established after the standard sample. Sperm suspensions were
202 analysed in duplicate in a Gallios™ flow cytometer (Beckman Coulter, Bromma,
203 Sweden) equipped with standard optics: a violet laser (405 nm) with 2 colours, argon
204 laser (488 nm) with 5 colours, and a HeNe-laser (633 nm) with 3 colours. The filter
205 configuration was as follows: Blue: FL1 550SP 525BP and FL3 655SP 620/30. The
206 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/sec. After further calculations of the raw.fcs data in R-environment (package rflowcyt – Bioconductor), to obtain both %DFI (DNA Fragmentation Index above 24.5 %; DFI), ratio of red/red + green fluorescence, where red is broken DNA and green is native DNA, and %HDS (High DNA Stainability; dependent of the green fluorescence; HDS), 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.

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 forms 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 forms a large halo (i.e. the DNA loops largely disperse). Following the manufacturer protocol, 50 µL of the thawed sperm sample were 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 7 additional minutes. Finally, the excess of staining solutions was removed and the slides allowed to dry at room temperature. Visualization was performed in an inverted bright field microscope (LEICA D100, Stockholm, Sweden). A minimum of 200 spermatozoa per sample were scored at x400 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 ($\times 100$). 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 $\times 100$) 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 $\pm$ 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 significance statistical level of $P < 0.05$ was used.

257

258 **3 Results**

259 The conventional WHO-semen parameters did not statistically differ between the
260 idiopathic infertile patients and fertile donors. The coefficient of variation (CV)
261 between the replicate analyses was 3.6% for the SCSA and 4.7 % for the HaloSpermG2.
262 The proportions of spermatozoa with DNA fragmentation in fertile donors and patients
263 were similarly measured by either technique. For fertile semen donors, the Sperm
264 Chromatin Dispersion (SDF) was $20.9\% \pm 9.4$ (range: 6.8-41.0; CV= 44.9%; 95%
265 confidence interval (CI= 15.976, 25.824) and the DNA fragmentation after SCSA (DFI)
266 was $17.1\% \pm 7.8$ (range: 6.9-31.2; CV= 45.6%; CI= 13.014, 21.186). The patients
267 depicted an SDF of $40.1\% \pm 9.0$ (range: 24.6-59.6; CV= 22.4%; CI= 37.378, 42.822)
268 versus a DFI of $40.2\% \pm 19.3$ (range: 2.3-80; CV= 48.0%; CI [34.363, 46.037]). Values
269 did not differ statistically within group (ns), but the fertile semen donors had
270 significantly ($p<0.05$) fewer spermatozoa with fragmented DNA measured with either
271 method than patients with a diagnosis of idiopathic infertility. After the spermatozoa
272 were selected by colloid discontinuous gradient in patients, their sperm motility
273 increased to $89.7\pm 14.0\%$ and sperm velocity to $43.4\pm 11.9 \mu\text{m}/\text{sec}$ (Mean $\pm$ SD) and
274 those depicting fragmented DNA were significantly fewer ($p<0.05$) (SDF: $10.0\% \pm 5.9$
275 ($2.3/30.9$)) and DFI: 11.9 ± 11.2 ($0.6/53.5$)) compared to the initial ejaculate, posing a
276 tendency ($p>0.05$) to reach fertile donor values.

277

278 Lower values of “High DNA Stainability” (HDS), a measure (%) of the proportion of
279 immature spermatozoa having defects in the histone-to-protamine transition, were
280 found in samples from patients after the colloid discontinuous gradient ($6.7\% \pm 4.6$

(2.4/22)) compared to the initial ejaculates; either from patients ($13.2\% \pm 10.3$ (1.6/42.2)) or from fertile donors ($14.1\% \pm 10.2$ (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-Almand 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 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 DNA fragmentation index (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 (High DNA Stainability). 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 fertilization failure, low blastocyst development or no pregnancy (Evenson, 2017; Virro *et al.*, 2004;). The SCSA analyses large numbers of spermatozoa under short time, 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 *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 deproteinization 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 (TUNEL), the acridine orange test (AOT), the tritium-labelled 3H-actinomycin D (3H-AMD) incorporation assay, the *in situ* nick translation (ISTN), the DNA breakage detection-fluorescence *in-situ* hybridizations (DBD-FISH), 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, is 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: $17.1\% \pm 8.0$; SDF: $20.9\% \pm 9.4$). 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% confidence intervals 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 *et al.*, 2006a). The semen from the fertile donors used for IVF have 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 three-fold 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 fertilization, 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, e.g. $<24.5\%$ (Mean $\pm$ SD (min/max)): No pregnancy-to-term (DFI: $9.6\% \pm 8.4$ (0.6-34.4) vs. SDF: $8.7\% \pm 6.0$ ((2.3-30.9)) vs. pregnancy-to-term (DFI: $12.9\% \pm 5.1$ (6.6-22.4) vs. SDF: $17.3\% \pm 14.5$ (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: fertilization, 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 *et al.*, 2002; Evenson, 2017) 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; Erenpreis, Spano, Erenpreis, Bungum & Giwerzman, 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.

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430 **Conflict of interest**

431 The authors declare no conflicts of interest to disclose.

432

433 **Authors' Contributions**

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

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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