Archives of Medical Research 55 (2024) 103122

ORIGINAL ARTICLE

Strong Correlation Between Double-Strand DNA Breaks and Total Sperm DNA Fragmentation in the Human Ejaculate

Jaime Gosálvez,[a] Stephen D. Johnston,[b,c,d] Ahinoa Prado,[c,d] Carmen López-Fernández,[a] Pablo Contreras,[c,d] Javier Bartolomé-Nebreda,[a,c,d] Mercedes González-Martínez,[e] José Luis Fernández,[f,g] Carlos García de la Vega,[a] and Alfredo Góngora[h]

[a] *Department of Biology, Universidad Autónoma de Madrid, Madrid, Spain*
[b] *School of Environment, University of Queensland, Gatton, Queensland, Australia*
[c] *School of Veterinary Science, University of Queensland, Gatton, Queensland, Australia*
[d] *Halotech DNA, Calle Faraday SN, Parque Científico de Madrid, Madrid, Spain*
[e] *GINEMED Clinic, Farmacéutico Murillo Herrera, Sevilla, Spain*
[f] *Unidad de Genética, Complejo Hospitalario Universitario A Coruña, Instituto de Investigación Biomédica de A Coruña, A Coruña, Spain*
[g] *Laboratorio de Genética Molecular y Radiobiología, Centro Oncológico de Galicia. A Coruña, Spain*
[h] *Centro de Fertilidad, Mexico City, Mexico*

Received for publication May 18, 2024; accepted October 30, 2024 (ARCMED-D-24-00380).

Background. Double- and single-strand DNA breaks (DSBs and SSBs, respectively) in spermatozoa, which emerge from intrinsic and extrinsic degenerative processes, are likely related to the underlying male pathology.

Aim. To determine whether the incidence of DSBs in the human ejaculate is a consistent predictor of whole sperm DNA fragmentation (W-SDF = SSBs + DSBs).

Methods. A correlation between the proportion of spermatozoa that showed whole W-SDF and those displaying only DSBs in DNA. Two patient cohorts were established: W-SDF ≤30% (low SDF; $n = 153$) and W-SDF ≥30% (high SDF; $n = 222$).

Results. An increasing level of W-SDF is associated with an increased incidence of DSBs in the ejaculate. When data from both the low and high W-SDF groups were combined, a linear relationship was observed, with DSBs increasing by 0.799 units for each unit increase in W-SDF. However, when the cohorts were analyzed separately, the relationships differed. In the low SDF group, DSBs increased linearly by 0.559 units for each unit increase in W-SDF. In the high SDF group, DSBs increased exponentially by 0.602 units per unit of W-SDF. Furthermore, the data dispersion between the two variables was significantly different between the cohorts, with the high SDF group showing 0.8 times greater variability than the low SDF group.

Conclusions. While the presence of DSBs in sperm is correlated with the W-SDF present in raw semen samples, the biological mechanisms responsible for DSBs are expressed in different proportions and/or at different levels in ejaculates with higher levels of DNA damage.

Key Words: Sperm DNA damage, Sperm DNA fragmentation, Double-stranded, DNA breaks, Single-stranded DNA breaks, Human ejaculates.

Address reprint requests to: Jaime Gosálvez, Department of Biology, Universidad Autónoma de Madrid, Ciudad Universitaria de Cantoblanco, 28049 Madrid, Spain; E-mail: jaime.gosalvez@uam.es

https://doi.org/10.1016/j.arcmed.2024.103122

Introduction

Loss of the orthodox conformation of the sperm DNA molecule resulting in the presence of both single- and/or double-strand DNA breaks (SSBs and DSBs, respectively) may be associated with intrinsic factors related to sperm production or extrinsic etiologies related to micro- and macro-environmental causes associated with each individual (1,2). SSBs are discontinuities in one strand of the DNA double helix and are often associated with damaged or mismatched 5′- and/or 3′-termini, whereas DSBs involve simultaneous breakage of both strands of the DNA double helix. Both types of breakage can be caused by a variety of direct and indirect stressors, such as oxidative stress, ionizing radiation, exposure to environmental toxins, errors during DNA replication, high temperatures, and enzymatic activity. The pre)sence of SSBs and DSBs in sperm DNA has been shown to compromise reproductive outcomes (3). In some cases, spermatozoa with either or both SSBs and DSBs that fertilize the oocyte following natural conception or using IVF/ICSI may be repaired, more or less efficiently, by the oocyte's DNA repair machinery (4), but this is not always a "foolproof" system.

There are multiple consequences associated with the presence of unrepaired DSBs and/or SSBs. For example, mutations may occur during DNA replication before fertilization if the replication fork encounters SSBs, misincorporates nucleotides, or skips damaged regions. This can result in base substitutions, insertions, or deletions in newly synthesized DNA strands. SSBs can hinder or abolish DNA replication by preventing the normal progression of the replication machinery. When DSBs occur on both strands at spatially separated sites, chromosomal rearrangements are formed when the broken ends are incorrectly rejoined, resulting in translocations, inversions, or deletions of the genetic material (5). SSBs can activate DNA damage response mechanisms, including DNA repair pathways such as base excision repair (BER) and nucleotide excision repair (NER). When DSBs are present, alternative DNA repair mechanisms are activated. For example, non-homologous end joining (NHEJ), which directly ligates broken ends, and homologous recombination (HR), which uses an intact homologous DNA template for repair, operate in the presence of DSBs (6). Finally, both SSBs and DSBs can trigger cell cycle checkpoints that may temporarily undergo programmed cell death or enter a senescent state to prevent the propagation of potentially damaged DNA (7).

While the impact of SDF on human reproduction (both SSBs and DSBs) has been widely investigated (8), knowledge of the specific influence of SSBs and DSBs as independent events on reproductive outcomes is limited. These studies are not easy to perform, given the limited technology available to differentiate the true presence of SSBs, DSBs, or a combination of both events affecting the same sperm cell (9). DSBs can be characterized using a neutral comet assay (10) or a specific chromatin dispersion test (11); both methodologies were conducted using native chromatin in sperm cells processed under neutral pH conditions to avoid DNA denaturation so that only double-stranded DNA chains were displaced during electrophoresis. SSBs and DSBs are susceptible to denaturation under mild acid or alkaline conditions. This results in single-stranded DNA stretches that can be moved or dispersed during electrophoresis and chromatin dispersion tests, respectively. Thus, the use of DNA denaturation-based methodologies can provide information on the proportion of total (SSBs + DSBs) DNA breaks present in the sperm DNA, while the absence of DNA denaturation provides information on the presence of DSBs.

This study aimed to determine whether there was a correlation between the level of whole DNA fragmentation (W-SDF: DSBs + SSBs) in spermatozoa and the incidence of DSBs in the same human ejaculate. Sperm DNA fragmentation is a multifactorial event, and in the absence of known patient etiology, the most parsimonious and intuitive hypothesis is that the level of DSBs present in the ejaculate will be positively correlated with the W-SDF present in the semen sample.

Materials and Methods

Patient Cohorts

This prospective study analyzed a cohort of 375 men between 24 and 45 years of age who presented for diagnostic evaluation at their first semen analysis at a reproductive health clinic in Seville, Spain. The study spanned 4 years, from 2020 to 2023, and was part of a larger blind study on various aspects of SDF. Inclusion and exclusion criteria were rigorously applied to refine the study population. Exclusion criteria included patients with severe oligospermia (sperm concentration less than two million spermatozoa per milliliter), asthenozoospermia (less than 30% motile spermatozoa), severe teratozoospermia (≤4% of spermatozoa in the ejaculate meeting morphological normality), individuals diagnosed with or receiving treatment for cancer, cases of pronounced leukocytopenia, varicocele, paraplegia or a diagnosis of COVID-19. All patient data included in this study had patient consent and ethical approval from both the clinic where the samples were collected (Clínica Ginemed, Unidad de Reproducción, Sevilla) and the Human Ethics Committee of the University of Seville (approval number - 3375125c8b5f1d04c9511825aef98d309135328c).

Sperm DNA Analysis

All individuals were processed to simultaneously assess the presence of W-SDF and DSBs based on the same ejacu-

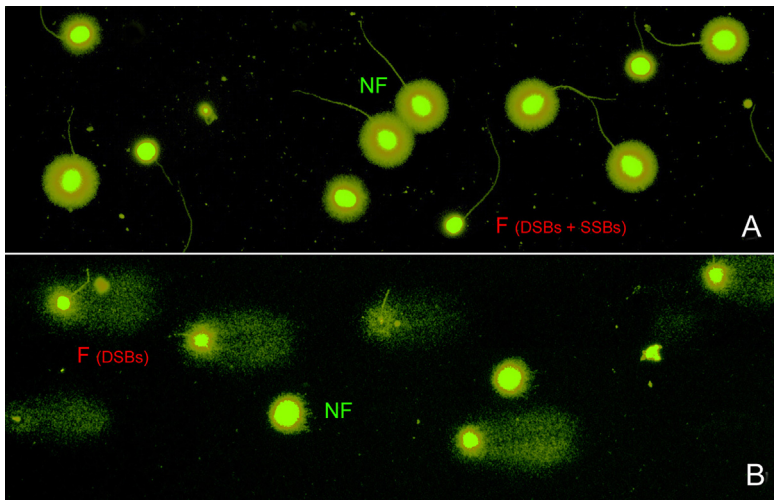

Figure 1. Sperm DNA fragmentation as visualized with the sperm chromatin dispersion (SCD) test A, and the neutral comet assay, B. The original figures have been electronically filtered to enhance regional differences in chromatin density. F – sperm with fragmented DNA; NF – sperm with no fragmented DNA; DSBs – double-strand breaks; SSBs – single-strand breaks.

lation. For statistical purposes, the data were processed and considered as paired samples of raw samples obtained after masturbation. To homogenize the impact of any iatrogenic damage on sperm DNA, patients were asked to abstain for 2 d, and all samples were processed 30 min after liquefaction. SDF was assessed using the Halosperm GII kit (Sperm Chromatin Dispersion test; Halotech DNA, Madrid, Spain) according to the manufacturer's instructions. This system provides differential sperm morphology after treatment, in which spermatozoa showing a large or medium halo of dispersed chromatin represent those without DNA fragmentation (Figure 1A), whereas spermatozoa showing a small or absent halo around a well-defined core are classified as containing fragmented DNA (Figure 1B). A total of 300 spermatozoa were evaluated per slide. The presence of DSBs, visualized by a neutral comet assay, was assessed as previously described (9). After slide processing, spermatozoa with comet tail displacement greater than half the halo size were considered to have DNA damage (Figure 1B). Slides were stained with Fluorogreen (Halotech DNA, Madrid, Spain). All images were visualized and captured using a Nikon Eclipse microscope equipped with a high-resolution Nikon 12-bit CzCD (Nikon DS-Q) and using a 40x fluorite objective. For each patient, the sperm chromatin dispersion test (SCDt) provided data on the proportion of total SDF (SSB + DSB) in the ejaculate, while the comet assay provided comparative data on the proportion of DSB in the same semen sample.

Experimental Design

Once the database was generated using the data obtained on the presence of W-SDF, two patient cohorts were established: a threshold SDF value of 30% or less (low W-SDF) and another cohort of individuals with W-SDF higher than 30% (high W-SDF). As previously suggested by Gosálvez J, et al. (12) and Esteves SC, et al. (8), the 30% SDF threshold was considered critical to discriminate individuals facing fertility challenges. A correlation analysis of the corresponding DSB values (high and low) was then performed for each W-SDF cohort (high and low), together with a similar correlation analysis of W-SDF and DSBs.

Statistics

Descriptive and analytical statistics were performed using the original dataset on Microsoft Excel files exported to SPSS (IBM SPSS v25 Statistics Package, NY, USA). Normality tests for data distribution in the different groups were performed using the Kolmogorov-Smirnov test. Accordingly, non-parametric statistics for paired or independent groups were used to compare the values in different groups. The Wilcoxon signed-rank and the Mann-Whitney U (confidence interval $\alpha = 0.05$) two-tailed tests were applied. The Spearman Rho was used to assess the correlation analysis, and the F test was used to compare the variances of the two samples. Finally, we used the mean signed difference (MSD) as a measure of central tendency, which represents the average of the signed (positive or negative) differences between paired values (13). This was used to analyze the data dispersion between the values observed for W-SDF and DSBs when the individuals were classified according to a low level of SDF (less than 30%) or higher. In this case, due to the structure of the data, the MSD is represented by the absolute values of the difference of the paired values for W-SDF and DSBs.

Table 1. Descriptive statistics for whole sperm DNA fragmentation (W-SDF) and double-strand DNA breaks (DSBs) across two defined cohorts based on SDF values that were <30, and >30%

	Total cohort population		Low SDF		High SDF	
	W-SDF	DSBs	W-SDF	DSBs	W-SDF	DSBs
N	375	375	153	153	222	222
Mean	37.1	29.8	20.1	15.0	48.8	40.0
Median	34.0	26.0	20.0	14.3	46.0	38.0
Range	83.3	87.6	23.9	25.3	59.3	79.6
CI 95%	35.3–39.0	28.0–31.5	19.2–21.1	14.0–16.0	46.9–50.8	38.1–41.9
IQ	26.7	26.6	10.9	8.6	21.9	21.35

N, Sample size; CI, Confidence interval, indicating the range within which the true mean of the population was expected to lie with a probability of 95%; IQ: Interquartile range, representing spread of the middle 50% of the data points.

Results

W-SDF versus DSBs

The differences between the presence of W-SDF and DSBs in the same ejaculate for all patients were analyzed and the results are shown in Table 1 and Figure 2A. As the data in both groups did not conform to a normal distribution (W-SDF: Kolmogorov-Smirnov = 0.086, df 375, p <0.000; DSBs: Kolmogorov-Smirnov 0.101, df 375, p <0.000), non-parametric paired sample statistics were used to compare groups. This analysis revealed that W-SDF was significantly higher than the DSBs (Wilcoxon Z = –14.88, bilateral p <0.000). A correlation analysis using both variables (W-SDF and DSBs) is shown in Figure 2B, which revealed a strong relationship between both variables (Spearman's rho = 0.908, bilateral p <0.000). To better understand the relationship between the W-SDF and DSBs, regression analysis was performed considering linear, logarithmic, and exponential models. For this analysis, W-SDF was considered as the dependent variable and DSBs as the independent variable. The linear model yielded the highest R^2 value of 0.799, such that for every unit increase in W-SDF, DSBs increased by an average of 0.799 units; this effect was highly significant (F = 1482.1, p <0.000).

W-SDF versus DSBs in Individuals with Low W-SDF

The datasets for low W-SDF (Kolmogorov-Smirnov = 0.104, df = 153, p <0.000) and low DSBs (Kolmogorov-Smirnov = 0.083, df = 153, bilateral p = 0.013) were not normally distributed so that non-parametric tests were used for the analysis. Descriptive information on the SDF levels in both groups is shown in Table 1 and Figure 2C. Low W-SDF was significantly higher than the low DSBs (Wilcoxon Z = –10.09, bilateral p <0.000). A correlation analysis between these two variables revealed a moderate to strong positive correlation (Spearman's rho = 0.739, bilateral p <0.000). To understand the relationship between low W-SDF and low DSBs, regression analysis considering linear, logarithmic, and exponential models was performed. For this analysis, low W-SDF was considered as the dependent variable and low DSBs as the independent variable. The linear model (Figure 2D) offered the highest R^2 value of 0.559, such that for every unit in which low W-SDF increased, low DSBs increased by an average of 0.559 units; this relationship was statistically significant (F = 191.03, p <0.000).

W-SDF versus DSBs in Individuals Presenting High W-SDF

Datasets for high W-SDF (Kolmogorov-Smirnov = 0.104, df 222, bilateral p <0.000) and high DSB (Kolmogorov-Smirnov = 0.0173, df = 222, p = 0.003) were not normally distributed, so that non-parametric tests were used for the analysis. Descriptive information on SDF levels in both groups is shown in Table 1 and Figure 2E. High W-SDF was significantly higher than high DSBs (Wilcoxon Z = –11.18, bilateral p <0.000). Correlation analysis between these two variables revealed a strong correlation (Spearman's rho = 0.782, bilateral p <0.000). Regression analysis was used to determine the relationship between high W-SDF and high DSBs, considering linear, logarithmic, and exponential models. For this analysis, high W-SDF was considered as the dependent variable and high DSB was the independent variable. In this case, the exponential model (Figure 2F) yielded the highest R^2 value of 0.602, such that for every unit in which high W-SDF increased, high DSBs increased by an average of 0.692 units; this effect was significant (F = 191.03; p <0.000).

Mean Signed Difference (MSD) Analysis

The MSD (mean signed difference) values used to statistically compare data dispersion between the high and low SDF cohorts are shown in Table 2. The data sets for low MSD (Kolmogorov-Smirnov = 0.107, df = 153, bilateral p <0.000) and high MSD (Kolmogorov-Smirnov = 0.112, df = 221, p = 0.000) were not normally distributed; therefore, non-parametric tests were used for the analysis. The mean values obtained in the MSD were approximately 0.8

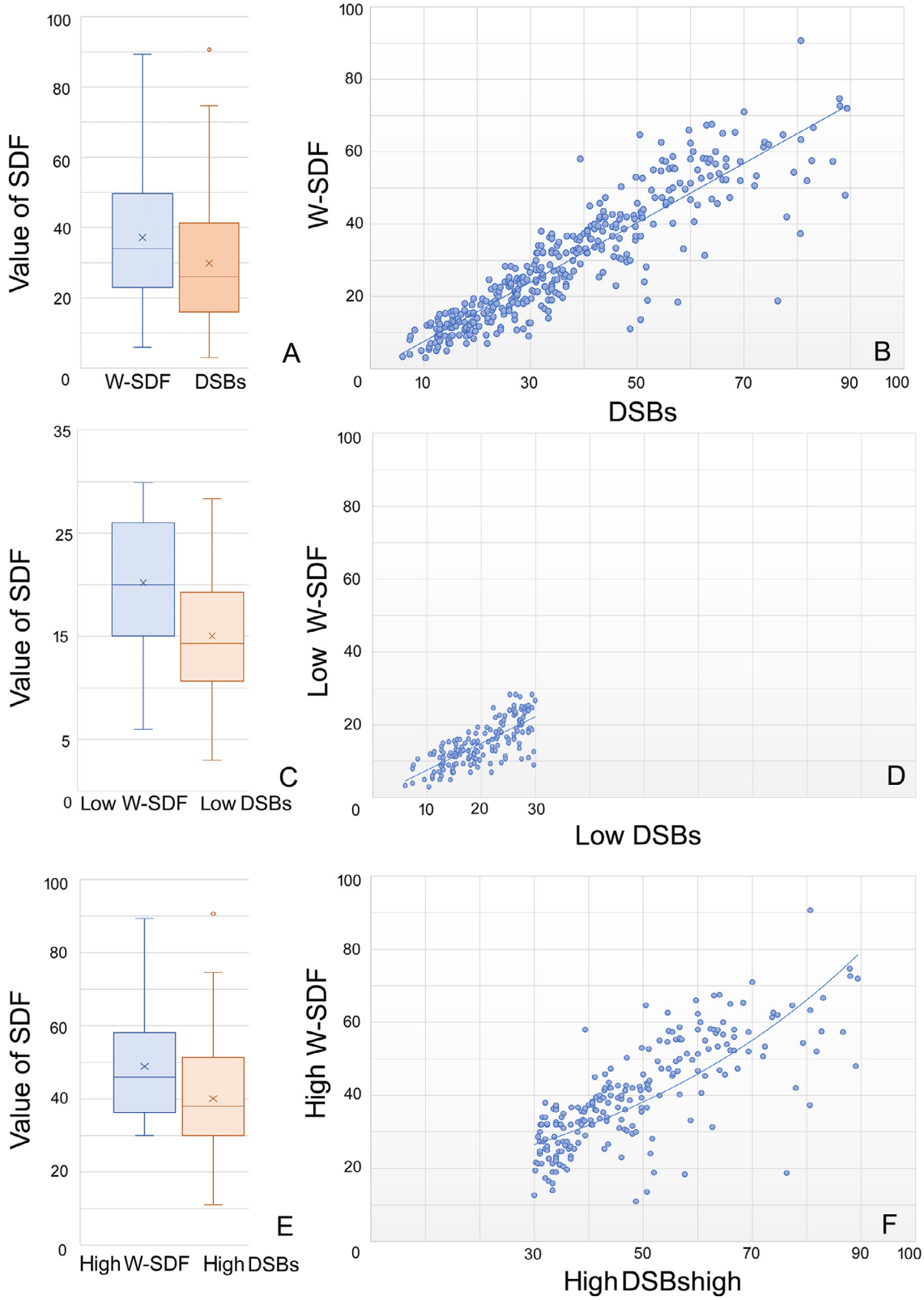

Figure 2. The relationship between whole sperm DNA fragmentation (W-SDF) and the incidence of DSBs (Double Strand Breaks). A, C, and E. Box and whisker diagrams showing the level of SDF when the W-SDF and DSBs are represented using A, the whole sample, C. samples with W-SDF values lower than 30%, and E, the sample with W-SDF values higher than 30%. B, D, and F. Correlation plots of W-SDF and DSBs B, of the whole sample, D, W-SDF values lower than 30%, and F, WDF values higher than 30%.

Table 2. Numerical representation of the MSD values obtained in groups with sperm DNA fragmentation lower than 30% (low W-SDF) and higher than 30% (high W-SDF)

	N	Mean	Median	Range	CI 95%	IQ
Low W-SDF	153	5.4	4.6	29.6	4.8–6.0	5.04
High W-SDF	222	8.2	7.0	37.5	7.1–9.2	8.04

N, Sample size; CI, Confidence interval, indicating the range within which the true mean of the population is expected to lie with a specified probability; *IQ*: Interquartile range, representing the spread of the middle 50% of the data points.

times higher in the high W-SDF group than in the low W-SDF group (Table 2); *i.e.*, the data dispersion of W-SDF with respect to DSBs was more pronounced in the high W-SDF cohort (Mann-Whitney $U = 11{,}564$; bilateral $p < 0.000$).

Discussion

As expected, the results obtained in this study clearly showed that the incidence of W-SDF was in all cases higher than the values observed for DSBs. In addition, it was observed that the higher the level of W-SDF in the ejaculate, the higher the incidence of DSBs. In general, when all patient sample analyses were plotted together, this increase conformed to a linear model, where it was found that for every unit in which the W-SDF increased, the DSBs also increased by an average of 0.80 units. However, the correlation was different when individual patients were separated into cohorts with W-SDF values lower or higher than 30%. When the low W-SDF cohort was analyzed separately, there was a linear pattern of increase in SDBs of 0.55 units per unit of W-SDF, whereas the high W-SDF cohort was best represented by an exponential pattern of increase in DSBs of 0.60 units for each high W-SDF increase.

The results obtained with the MDS values to assess the net difference in scores observed between W-SDF and DSBs also indicated that the dispersion of these values was two times higher in the high W-SDF cohort compared to that of the low W-SDF cohort. This finding suggests that in severely compromised ejaculates with high levels of W-SDF, the effectors leading to either SSBs or DSBs are not as balanced as in ejaculates with low levels of SDF. We suggest that the biological mechanisms responsible for DSBs, such as defective protamination, apoptosis, or an unbalanced REDOX environment (14), are expressed in different proportions and/or levels of activity in ejaculates with higher levels of DNA damage, which in turn could be related to the specific type of pathology in each patient.

The observed disparity in the dispersion of the results when comparing the low W-SDF and the high SDF, could be related to the multifactorial origin of SDF. Interestingly, the number of molecular mechanisms that have been associated with DSB production is higher than those producing SSBs. Defective chromatin condensation can produce DSBs via defective DNA repair, resulting in abnormal protamine 1/protamine 2 ratios (15,16). DSBs may also occur via abortive apoptotic processes, where abnormal spermatozoa enter an apoptotic-like process, or even when sperm with damaged DNA escape the Sertoli cell screening system (17). Lipid oxidation has also been associated with DSBs (18). In contrast, the presence of SSB lesions has been associated with oxidative stress processes, leading to the formation of 8-OHG-8-hydroxyguanosine and 8-OHdG-8-hydroxygunanosine adducts (19). In any case, we must consider DNA abnormalities that produce SSBs as well as other structural modifications involving cell membranes, mitochondria, proteomics, transcriptomics, metabolomics, lipidomics, epigenomics, and all processes with the capacity to modify the general homeostasis of the cell, may cause targeted sperm cells to enter an apoptotic state, which may ultimately lead to the production of DSBs.

The presence and activity of apoptotic signals in human sperm cells in response to different stimuli have been well documented in the literature (20,21). We also know that the DNA damage observed in the sperm nucleus is not static but a dynamic condition (22,23). Minor non-orthodox changes in the chromatin/DNA may trigger alternative mechanisms to increase the level of DNA damage. In the case of spermatozoa, it is widely known that one of the main processes associated with apoptosis is the presence of phosphatidylserine in the membrane with subsequent caspase activation (24). It has been demonstrated that internal cellular signals, such as DNA damage, unbalanced REDOX environment, or loss of cell survival signals, can activate pro-apoptotic proteins, such as BAX and BAK, and inhibit anti-apoptotic proteins, such as BCL-2 and BCL-xL (25,26). Cytochrome c, together with Apaf-1, which is considered an apoptotic protease-activating factor-1 and procaspase-9, forms a complex called the "apoptosome", which controls the onset of many known forms of intrinsic apoptotic processes in mammals (27,28). Caspases such as caspase-3, −6, and −7 can activate caspase-9, with the subsequent cleavage of various cellular proteins to induce cell death (29).

All of these factors associated with cell death can be considered intrinsic pathways. However, there is another set of extrinsic pathways that make the phenomenon and mechanisms of cell death much more difficult to unravel. For example, external signals that bind to cell surface death receptors, such as Fas (CD95) and TNF receptor 1 (TNFR1), in addition to the binding of ligands such as Fas ligand (FasL), may also be involved in cell death (30). In general, the production of these adaptor proteins facilitates the activation of procaspase-8, leading to death-inducing signaling. Finally, crosstalk between the intrinsic and extrinsic pathways may amplify these apoptotic

signals (31,32). Probably, some of these mechanisms are triggered when the spermatozoa are exposed to different insults, which consequently alter the ratio of single- to double-stranded breaks.

Many of the DSBs present in sperm are generated during meiosis (33,34) and, if unrepaired, can reach the developmental stage of mature sperm cells (35). In addition, the accumulation of extra DSBs during apoptosis greatly increases the number of DSBs in maturing sperm cells. Impairment of signaling and DNA repair during spermiogenesis may result in persistent DSBs in mature spermatozoa. Given this complex and dynamic scenario, and in an attempt to explain the higher presence of W-SDFs compared to DSBs, the cells presenting with SSBs may ultimately be identified by some of these extrinsic or intrinsic pathways to produce cell death that culminates in the production of DSBs. Thus, the presence of different types of DNA damage associated with an individual must be considered as a dynamic and transient expression with a complex scenario of newly produced DNA breaks that probably start as SSBs and then degenerate into DSBs and cell death. The intensity of each effector, or the synergistic combination of different effectors, may also be critical in modulating the presence of different types of DNA damage. While the pathways and mechanisms underlying cellular apoptosis are multifaceted when a cell is exposed to and impacted by multiple structural and functional perturbations, the integrity of the DNA molecule is compromised.

The information provided by the MSD data analysis highlights the difference in data dispersion observed when the W-SDF was plotted against DSBs. MSD values were significantly higher in individuals with high W-SDF than in those with low W-SDF. This suggests that for a fixed value of the W-SDF, the value of the DSBs tended to be less associated with the W-SDF in the group presenting with SDF levels above 30% than when the W-SDF level was below 30%. This observation is consistent with another report by our group, which analyzed the variation in the level of W-SDF, after comparing different ejaculates in individuals presenting with SDF lower or higher than 30% (13). In this study, it was also observed that the higher the value of W-SDF dispersion, the higher the level of W-SDF. This indicates that W-SDF above 30% accumulates a series of profiles related to compromised DNA stability, which is congruent with poor male fertility, and these variations may be associated with the unique etiology of a dysfunctional sinogram.

Nevertheless, the preliminary information presented in the current study needs to be explored in more detail, and it seems plausible that different etiologies associated with male infertility may present a specific incidence of W-SDF over both DSBs and SSBs; this is indeed the case in patients with spinal cord injury (SCI), who present extreme values of W-SDF, exceeding a level higher than 70% (36). In patients with SCI, ejaculate levels of W-SDF were 3.3 times higher than those observed in controls with SDF lower than 30%. These patients had a median DSB value of approximately 57%, which was significantly higher than that observed in normozoospermic patients (median = 4.6%). In contrast, the proportion of SSBs in patients with SCI was significantly higher in normozoospermic ejaculates than in men with paraplegia (9). The presence of high SSB levels in normozoospermic individuals was also reported by Tímermans et al. (11). In the case of SCI patients, this probably indicates that many spermatozoa containing SSBs in the early stages of their development accumulate DSBs due to the long periods of anejaculation inherent to these individuals.

Another example that illustrates the specific potential manifestation of SSBs or DSBs is when patients are diagnosed with varicocele. These individuals have a significantly elevated proportion of spermatozoa with a degraded DNA molecule, as evidenced by the sperm chromatin dispersion test (37). This suggests that a subpopulation of spermatozoa is characterized by extensive DNA damage, including both substantial double-stranded and single-stranded DNA breaks. In the case of patients with varicocele, the prevalence of degraded spermatozoa was observed to be at least twice as high in infertile men with varicocele as in fertile controls. Furthermore, this prevalence was also increased compared to men with other infertility pathologies (37).

Conclusions

From a clinical perspective, although there is a tendency for W-SDF values to be slightly higher than those reported for DSBs, the high correlation between both variables suggests that SDF assessment by either W-SDF (SCD) or DSBs (comet assay) essentially results in relatively equivalent outcomes. However, the relative differences between these two methods need to be analyzed in a variety of different pathological scenarios, as the differentiation of DSBs from SSBs may potentially reflect or provide insight into the specific underlying etiology.

Acknowledgments

We would like to thank all the members of the research team at the UAM for their willingness to participate in the study. We would like to thank the personnel at Ginemed Clinic for their help in recruiting all the patients and Halotech for their technical support.

Conflict of Interest

The authors report no competing interests.

References

1. Ward JF. The complexity of DNA damage: relevance to biological consequences. Int J Radiat Biol 1994;66:427–432. doi:10.1080/09553009414551401.
2. Tamanoi F, Yoshikawa K. Overview of DNA damage and double-strand breaks. Enzymes 2022;51:1–5. doi:10.1016/bs.enz.2022.08.001.
3. Agarwal A, Barbăroşie C, Ambar R, et al. The impact of single- and double-strand DNA breaks in human spermatozoa on assisted reproduction. Int J Mol Sci 2020;21:3882. doi:10.3390/ijms21113882.
4. Newman H, Catt S, Vining B, et al. DNA repair and response to sperm DNA damage in oocytes and embryos, and the potential consequences in ART: a systematic review. Mol Hum Reprod 2022;28:gaab071. doi:10.1093/molehr/gaab071.
5. Caldecott KW. DNA single-strand break repair. Exp Cell Res 2014;15:2–8. doi:10.1016/j.yexcr.2014.08.027.
6. Huang R., Zhou P.K. DNA damage repair: historical perspectives, mechanistic pathways and clinical translation for targeted cancer therapy. Sig Transduct Target Ther 2021;6:254. 10.1038/s41392-021-00648-7.
7. Jasin M, Rothstein R. Repair of strand breaks by homologous recombination. Cold Spring Harb Perspect Biol 2013;1:a012740. doi:10.1101/cshperspect.a012740.
8. Esteves SC, Zini A, Coward RM, et al. Sperm DNA fragmentation testing: summary evidence and clinical practice recommendations. Andrologia 2021;53:e13874. doi:10.1111/and.13874.
9. Gosálvez J, Vargas-Baquero E, López-Fernández C, et al. Sperm DNA damage in men with spinal cord injury: the relative incidence of single- and double-strand DNA breaks. Androl 2022;10:1292–1301. doi:10.1111/andr.13210.
10. Clementi E, Garajova Z, Markkanen E. Measurement of DNA damage using the neutral comet assay in cultured cells. Bio Protoc 2021;20:e4226. doi:10.21769/BioProtoc.4226.
11. Tímermans A, Vázquez R, Otero F, et al. DNA fragmentation of human spermatozoa: simple assessment of single- and double-strand DNA breaks and their respective dynamic behavioral response. Androl 2020;8:1287–1303. doi:10.1111/andr.12819.
12. Gosálvez J, López-Fernández C, Fernández JL, et al. Unpacking the mysteries of sperm DNA fragmentation: ten frequently asked questions. J Reproductive Biotechnol Fertil 2015;4:1–16. doi:10.1177/2058915815594454.
13. González-Martínez M, López-Fernández C, Sánchez-Martín P, et al. Intra-individual variation of sperm DNA fragmentation in the human ejaculate. Syst Biol Reprod Med 2023;69:215–222. doi:10.1080/19396368.2023.2175738.
14. Muratori M, Tamburrino L, Marchiani S, et al. Investigation on the origin of sperm DNA fragmentation: role of apoptosis, immaturity, and oxidative stress. Mol Med 2015;21:109–122. doi:10.2119/molmed.2014.00158.
15. García-Peiró A, Martínez-Heredia J, Oliver-Bonet M, et al. Protamine P1/P2 ratio correlates with dynamic aspects of DNA fragmentation in human sperm. Fertil Steril 2011;95:105–109.
16. Kuchakulla M, Narasimman M, Khodamoradi K, et al. How defective spermatogenesis affects sperm DNA integrity. Andrologia 2021;53:e13615. doi:10.1111/and.13615.
17. Aitken RJ, Koppers AJ. Apoptosis and DNA damage in human spermatozoa. Asian J Androl 2011;13:36–42. doi:10.1038/aja.2010.68.
18. Yang S, Hwang S, Kim B, et al. Fatty acid oxidation facilitates DNA double-strand break repair by promoting PARP1 acetylation. Cell Death Dis 2023;14:435. doi:10.1038/s41419-023-05968-w.
19. Singh A, Kukreti R, Saso L, et al. Oxidative stress: role and response of short guanine tracts at genomic locations. Int J Mol Sci 2019;20:4258. doi:10.3390/ijms20174258.
20. Espino J, Mediero M, Lozano GM, et al. Reduced levels of intracellular calcium releasing in spermatozoa from asthenozoospermic patients. Reprod Biol Endocrinol 2009;7:1–11.
21. Monllor F, Espino J, Marchena AM, et al. Melatonin diminishes oxidative damage in sperm cells, improving assisted reproductive techniques. Turk J Biol 2017;41:881–889.
22. Gosálvez J, López-Fernández C, Fernández JL, et al. Relationships between the dynamics of iatrogenic DNA damage and genomic design in mammalian spermatozoa from eleven species. Mol Reprod Dev 2011;78:951–961.
23. López-Fernández C, Crespo F, Arroyo F, et al. Dynamics of sperm DNA fragmentation in domestic animals II. The stallion. Theriogenology 2007;68:1240–1250.
24. Fadok VA, Voelker DR, Campbell PA, et al. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J Immunol 1992;148:2207–2216.
25. Wolf P, Schoeniger A, Edlich F. Pro-apoptotic complexes of BAX and BAK on the outer mitochondrial membrane. Biochimica et Biophysica Acta Mol Cell Res 2022;1869:119317. doi:10.1016/j.bbamcr.2022.119317.
26. Qian S, Wei Z, Yang W, et al. The role of BCL-2 family proteins in regulating apoptosis and cancer therapy. Front Oncol 2022;12:985363. doi:10.3389/fonc.2022.985363.
27. Shaha C, Tripathi R, Mishra DP. Male germ cell apoptosis: regulation and biology. Philos Trans R Soc B Biol Sci 2010;365:1501–1515.
28. Zhou M, Li Y, Hu Q, et al. Atomic structure of the apoptosome: mechanism of cytochrome c- and dATP-mediated activation of Apaf-1. Genes Dev 2015;29:2349–2361. doi:10.1101/gad.272278.115.
29. Brentnall M, Rodriguez-Menocal L, De Guevara RL, et al. Caspase9, caspase-3, and caspase-7 have distinct roles during intrinsic apoptosis. BMC Cell Biol 2013;9:32 32. doi:10.1186/1471-2121-14-.
30. Guicciardi ME, Gores GJ. Life and death by death receptors. faseb J 2009;23:1625–1637. doi:10.1096/fj.08-111005.
31. Roy S, Nicholson DW. Cross-talk in cell death signaling. J Exp Med 2000;16(192):F21–F25.
32. Elmore S. Apoptosis: a review of programmed cell death. Toxicol Pathol 2007;35:495–516. doi:10.1080/01926230701320337.
33. Dernburg AF, McDonald K, Moulder G, et al. Meiotic recombination in C. elegans is initiated by a conserved mechanism and is dispensable for homologous chromosome synapsis. Cell 1998;94:387–398. doi:10.1016/s0092-8674(00)81481-6.
34. Symington LS. Mechanism and regulation of DNA end resection in eukaryotes. Crit Rev Biochem Mol Biol 2016;51:195–212. doi:10.3109/10409238.2016.1172552.
35. Qu W, Liu C, Xu YT, et al. The formation and repair of DNA double-strand breaks in mammalian meiosis. Asian J Androl 2021;23:572–579. doi:10.4103/aja202191.
36. Vargas-Baquero E, Johnston S, Sánchez-Ramos A, et al. The incidence and etiology of sperm DNA fragmentation in the ejaculates of males with spinal cord injuries. Spinal Cord 2020;58:803–810. doi:10.1038/s41393-020-0426-6.
37. Gosalvez J, Rodríguez-Predreira M, Mosquera A, et al. Characterization of a subpopulation of sperm with massive nuclear damage as recognized with the sperm chromatin dispersion test. Andrologia 2013;46:602–609. doi:10.1111/and.12118.