



ARTICLE

Free circulating DNA and DNase activity in the ejaculates of men with spinal cord injury

Javier Bartolomé-Nebreda^{1,2} · Eduardo Vargas-Baquero³ · Carmen López-Fernández^{1,2} · José Luís Fernández⁴ · Stephen Johnston⁵ · Jaime Gosálvez^{1,2}

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Abstract

Study design Retrospective descriptive study.

Objectives To study the presence of cell-free DNA (cfDNA) and DNase activity in males with spinal cord injury (SCI) with elevated sperm DNA fragmentation.

Setting Hospital in Toledo, Spain; University-based Genetics laboratory in Madrid, Spain.

Methods Semen was collected from 15 males with spinal cord injury and elevated sperm DNA fragmentation (SDF). The presence and concentration of cfDNA was assessed using standard gel electrophoresis and microfluidic electrophoresis. DNase activity was evaluated in seminal plasma and under the presence of EDTA and EGTA to control the response of enzyme activity. cfDNA fragments were mapped on the sperm genome using FISH. All results were compared to 15 normozoospermic fertile donors.

Results Standard gel electrophoresis revealed a cfDNA band of ~150 bp in all samples from males with SCI; this band was occasionally accompanied by another band of ~90 bp. These bands were not observed in normozoospermic donors. Microfluidic electrophoresis only identified the equivalent band of 150 bp. No correlation was observed between the intensity of the two bands and the level of SDF in males with SCI. Although DNase activity was present in the seminal plasma of both normozoospermic donors and men with SCI it did not digest cfDNA. cfDNA fragments were found to be hybridized all over the sperm genome.

Conclusions SCI patients with elevated sperm DNA fragmentation showed a 150 bp DNA band of cfDNA in the seminal plasma, which appeared resistant to DNase activity.

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✉ Stephen Johnston
s.johnston1@uq.edu.au

- ¹ Unit of Genetics, Department of Biology, Universidad Autónoma de Madrid, Madrid, Spain
- ² Halotech DNA, Cantoblanco, 28049 Madrid, Spain
- ³ Sexual and Fertility Unit, Hospital Nacional de Paraplégicos de Toledo, Toledo, Spain
- ⁴ INIBIC-Complejo Hospitalario Universitario A Coruña (CHUAC), Genetics Unit, As Xubias, 84, 15006-A Coruña, Spain
- ⁵ School of Agriculture and Food Sciences, University of Queensland, Gatton, QLD, Australia

Introduction

The localization and characterization of circulating cell-free DNA (cfDNA) is attracting increased scientific attention because of its clinical importance in being associated with a range of diseases [1]. cfDNA is detected in blood samples but it can also be identified in other body fluids [2, 3], associated with programmed cell death (apoptosis) or autolytic processes related with cell toxicity, infections or other cell insults concomitant with the unregulated digestion of cellular components [4]. Active release of certain components from viable cells (exosomes) as well as products related with the presence of neutrophil extracellular traps and mitochondrial DNA may also be considered as natural contributors of cfDNA [5, 6].

In parallel, the presence of deoxyribonuclease (DNase) has been found to be expressed and active in tissues and body fluids. Both, endogenous nucleases emerging from the

spermatozoa and exogenous seminal plasma nucleases have been described in the human ejaculate [7–10]. Given the capability of these enzymes to digest nuclear DNA in intact somatic cells [11], the elevated activity of endogenous nucleases has been linked to sperm DNA degradation [10]; it is therefore possible that DNase activity in seminal plasma may have an adverse effect on sperm DNA.

Males affected by spinal cord injury (SCI) have ejaculates of poor quality, characterized by low sperm motility, viability, abnormal sperm morphology and abnormal seminal plasma composition [12, 13]. In addition, the level of sperm DNA fragmentation (SDF) in ejaculates of males with SCI can be up to 5.4X the levels found in normozoospermic donors, with some SCI males showing close to 100% [14], a phenomenon which is likely to be connected with imperative anejaculation [15].

Given the extremely high levels of SDF in the ejaculates of males with SCI, the aim of the present investigation was to study the occurrence of cfDNA in these individuals and to compare these with that of normozoospermic donors of proven fertility. In parallel, we have also investigated the presence of DNase activity in the same ejaculates and the susceptibility of cfDNA to be digested with these enzymes under different conditions.

Material and methods

Male cohort description

Semen samples were obtained from 15 males with SCI (henceforth denoted “S” in all figures) attending the Sexual and Fertility Unit at the Hospital Nacional de Paraplégicos de Toledo, Spain. Males with SCI presented with either cervical (C) injury or thoracic (T) injury and according to the American Spinal Injury Association Impairment Scale (AIS; [16]). The degree of lesion completion was also noted and categorized as those with complete (A) or incomplete lesions (B, C and D). Descriptors of males with SCI that were included in the present study are given in Table 1. For a control comparison, fertile semen samples, surplus to requirements were analyzed from 15 normozoospermic donors without SCI (henceforth denoted “N” in figures). The mean (SD) age (years) of males with SCI and normozoospermic donors were 29.2 (7.04) and 23.4 (2.1), respectively.

Semen processing and experimental design

All semen samples from males with SCI in this study were obtained using vibro-stimulation (Ferticare Personal®, <https://medicalvibrator.com>) and a subsample of each ejaculate cryopreserved using a slow freezing protocol

Table 1 Descriptors of males with SCI included in the current study.

Patient	Age (years)	Lesion level	AIS scale	SDF
1	20	C5	B	88
2	23	T5	A	55
3	43	C6	A	65
4	31	C4	D	59
5	27	C4	B	85
6	25	T5	B	61
7	27	T4	A	63
8	20	T4	A	56
9	36	C7	B	65
10	25	T5	C	67
11	36	C5	C	77
12	27	T5	A	75
13	34	C6	A	67
14	23	C5	A	61
15	41	C6	A	51

Level of spinal injury: C—cervical vertebrate; T—thoracic vertebrate; A—complete spinal cord damage; B–D—incomplete spinal cord damage as defined by the International Standards for the Neurological Classification of SCI.

(Cryosperm, Origio, Malov, Denmark) for later assessment of SDF. None of the SCI men had children before their accident or were able to produce a pregnancy after their accident. SDF was evaluated using the Halosperm kit (Halotech DNA, Madrid, Spain; [14]).

As the aim of this study was to assess the presence and concentration of cfDNA and DNase activity and to determine whether there was a relationship with SDF, we selected frozen ejaculates from 15 males with SCI presenting with elevated levels of SDF (>50%) and compared these to 15 frozen ejaculates of normozoospermic donors that showed SDF levels <15% [17]. The majority of individuals (15 of 15 males with SCI and 10 of 15 normozoospermic donors) in the current study have been previously included in Vargas-Baquero et al. [14]. However, we used replicate samples so that a “fresh” value for SDF could be determined and to ensure that this value was directly related to corresponding levels of cfDNA and DNase activity evaluated in the same sample. The remaining ejaculates (five normozoospermic donors SDF < 15%) were from new individuals.

cfDNA isolation and fragment size determination

Semen samples were centrifuged at 600 × g for 10 min after which the supernatant was obtained and centrifuged again at 10,000 × g for 10 min to eliminate cellular debris. Clean seminal plasma was collected and stored at −20 °C until further analysis. For isolation of cfDNA, 50 µL of seminal

plasma was incubated with 50 μL of digestion buffer (20 mM Tris-ClH pH 7.5, 20 mM EDTA, 200 mM NaCl, 4% SDS, 40 μg proteinase K) for 2 h at 56 °C. After incubation, the digestion mixture was phenol extracted and the supernatant precipitated overnight at -20 °C with 100% ethanol. Finally, samples were centrifuged at 12,000 $\times g$ for 15 min and the DNA pellets were washed with 70% ethanol and resuspended in 10 μL of distilled H_2O . DNA concentration was measured by spectrophotometry (Eppendorf bioPhotometer; Eppendorf Ibérica, Madrid, Spain). For determination of cfDNA fragment size, 1 μL of each resuspended DNA sample was electrophoresed in 1% agarose gels in 1 \times TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.3) and stained with RedSafe (iNtRON Biotechnology, Seongnam-si, Gyeonggi-do, Korea).

DNA fragment length was also evaluated by microfluidic electrophoresis using the Agilent 2100 Bioanalyzer and DNA7500 chip (Agilent technologies Inc., Palo Alto, CA, USA). For this analysis only 12 of the 15 ejaculates from males with SCI and normozoospermic donors are reported.

Seminal plasma DNase activity

DNase activity in the seminal plasma was tested by incubating 4 μL of seminal plasma with 1 μL of total genomic DNA (0.5 ng μL^{-1}) for 15 min at 37 °C. After incubation, reaction volume was increased up to 15 μL by adding 10 μL of dH_2O before the mixture was phenol extracted. After centrifugation at 12000 $\times g$ for 15 min, aqueous phases were electrophoresed as described above. To test the effect of the inhibition of seminal plasma DNase activity on the presence of cfDNA, 4 μL of seminal plasma was incubated at 37 °C for 30 min with 0.5 μg of genomic DNA plus 10 mM EDTA, 10 mM EGTA or 10 mM EDTA plus 10 mM EGTA. After incubation, reaction mixtures were phenol extracted and the aqueous phases electrophoresed.

DNase I digestion

To analyze if the cfDNA in seminal plasma was protected from the action of seminal plasma nucleases, 0.5 μg of purified cfDNA and 8 μL of raw seminal plasma from a single male with SCI was digested with increasing amounts (0.1, 1, 5 and 10 IU) of recombinant DNase I (Thermo Fisher Scientific, Madrid, Spain) for 15 min at 37 °C. After digestion, samples were phenol extracted and the aqueous phases were electrophoresed. In an additional experiment, 0.5 μg of purified cfDNA and 8 μL of raw seminal plasma from a male with SCI was digested with 1 IU of recombinant DNase I for 5, 10, 15 and 20 min at 37 °C. After incubation, the digestion mixtures were phenol extracted and electrophoresed as indicated above.

DNA breakage detection—fluorescence in situ hybridization (DBD-FISH)

DBD-FISH was performed as described by Cortés-Gutierrez et al. [18] with slight modifications. Spermatozoa (50 μL) from three males with SCI were diluted in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , pH 7.0) at a concentration of 20×10^6 spermatozoa mL^{-1} and mixed with an equal volume of 1% low melting point agarose tempered at 37 °C. Subsequently, 20 μL of each mixture was deposited onto three pre-treated slides from the Halosperm Kit (Halotech DNA SL, Madrid, Spain) covered with a coverslip and allowed to solidify at 4 °C. After solidification, coverslips were removed and the slides were treated with the lysis solution of the Halosperm kit for 20 min at room temperature, followed by 10 min washing with 0.9% NaCl. To denature the DNA the slides were treated with 0.03 M NaOH 1 M NaCl (pH 12.5) for 2.5 min at room temperature. After DNA denaturation, the slides were neutralized with 0.4 M Tris-HCl (pH 7.5) for 5 min and washed in TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA; pH 8.3) for 2 min. Finally, the slides were dehydrated in sequential 70, 90 and 100% ethanol baths for 2 min each and air-dried. The DNA probe for hybridization was generated by labelling 1 μg of purified cfDNA with tetramethyl-rhodamine-5-dUTP (Merk, Madrid, Spain) using a commercial nick-translation kit (Roche Diagnostics, Madrid, Spain). For FISH, 100 ng of heat denatured cfDNA probe was deposited onto each slide and hybridized overnight at 37 °C in the dark. After hybridization, slides were washed in 2 \times SSC (Saline Solution Citrate: 0.3 M NaCl, 30 mM trisodium citrate; pH 7.0), dehydrated in sequential ethanol (70, 90, 100%) for 2 min each and air dried. Slides were counterstained with SYBR Green in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA).

Statistical analysis

Central tendency in this study has been reported as mean (standard deviation) but as not all data were normally distributed (Shapiro–Wilk test), median and 95% CI (lower and upper boundaries) are also reported. Comparison of means of parametric and non-parametric data were compared using a *t*-test and Kolmogorov–Smirnov Z test, respectively.

Results

SDF of males with SCI and normozoospermic donors

The results of SDF observed from males with SCI are shown in Table 1; the mean (SD) for the males with SCI

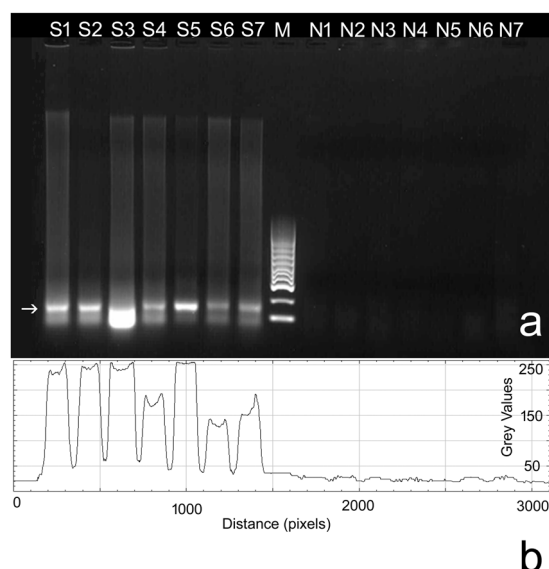


Fig. 1 Seminal plasma cfDNA isolated from males with SCI (S) and normozoospermic donors (N). (a) Gel electrophoresis M: 100 bp DNA ladder. (b) Surface profile shows intensity differences in the 90 bp band in SCI patients and normozoospermic donors.

and the normozoospermic donors were 66.8 (10.9) and 11.6 (2.0), respectively.

cfDNA seminal plasma levels and size in SCI patients and controls

Using standard gel electrophoresis and spectrophotometry, a DNA band of around 150 bp was observed in all cfDNA samples from males with SCI. In 11 out of the 15 cases this band was accompanied by another band of around 90 bp (Fig. 1, S1–S7). In general, the 150 bp band (arrow in Fig. 1) was more intense than the 90 bp; except in S3 that presented a prominent 90 bp band. Variability in the intensity of the bands in different individuals was noted (Compare S1 and S2 to S6 and S9 in Fig. 1); variability in the intensity was particularly evident in the 150 bp band (Fig. 1b). Equivalent molecular weighted bands were also present in normozoospermic individuals but at a lower intensity (Fig. 2, N1–N7). In terms of pixel intensity after electrophoresis (Fig. 1b), the level of fluorescence associated with the 90 bp band was 10X higher in males with SCI than in normozoospermic donors (Fig. 1b).

The mean concentration of cfDNA of males with SCI measured by spectrophotometry was 2.8X higher (Kolmogorov–Smirnov Z; $p = 0.000$) than that found in the normozoospermic donors [SCI males ($n = 15$): 0.1584 (SD: 0.0558) ng μL^{-1} ; range: 0.0778–0.2682 ng μL^{-1} and normozoospermic donors ($n = 15$): 0.0575 (SD: 0.0151) ng μL^{-1} ; range: 0.029–0.0816 ng μL^{-1}]. There was no correlation between the intensity of the two bands and SDF level in males with SCI. For example, S3 in Fig. 1 had an SDF value of

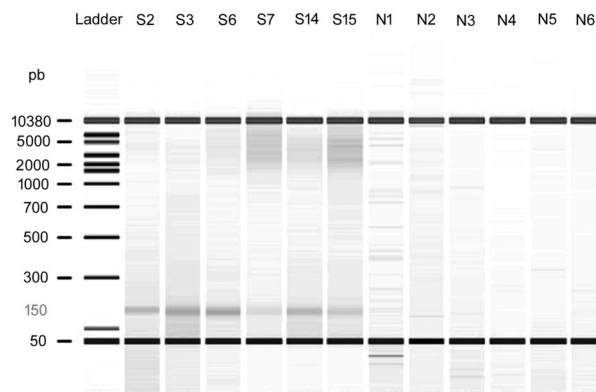


Fig. 2 High-resolution automated electrophoresis of DNA from the seminal plasma of 6 representative cases of males with spinal cord injury (S2–S15) and 6 normozoospermic donors (N1–N6).

65% (Table 1) and an intense band, whereas S6 and S7 had equivalent values of SDF (61% and 63%, respectively; Table 1) but possessed bands of less intensity (Fig. 1). DNA fluorimetry revealed that the cfDNA concentration of males with SCI ($n = 12$) had a higher mean (SD) concentration [8.01 (3.12) ng μL^{-1}] than normozoospermic donors ($n = 12$) [1.13 (0.51) ng μL^{-1}] and these results were consistent with corresponding spectrophotometry data.

When analyzed using high-resolution automated electrophoresis (Fig. 2), cfDNA fragments could be divided into 2 groups; (1) cfDNA fragments of low molecular weight (LMW: 70–300 bp) and (2) cfDNA fragments of high molecular weight (HMW: 800–9000 bp). To compare the seminal plasma of males with SCI to that of normozoospermic individuals with respect to their proportion of LMW and HMW cfDNA fragments, the data were examined for normality (See Table 2; Shapiro–Wilk Test). A non-parametric Kolmogorov–Smirnov Z test was used to compare the molecular weight of the HMW cfDNA fragments of the two groups but there was no difference (Table 2). Given the data for LMW cfDNA fragments were normally distributed, a *t*-test determined that the males with SCI had smaller fragments than normozoospermic donors (Table 2).

Of the two prominent bands observed using standard electrophoresis (150 and 90 bp) that characterized males with SCI, the only apparent band detected using the bioanalyzer was the one corresponding to 150 bp (Fig. 2; labelled in red).

DNase activity at the seminal plasma in SCI and controls

As the levels of cfDNA were higher in the seminal plasma of males with SCI, we tested whether this may be due to differences in DNase activity of the seminal plasma. Purified whole genomic DNA was incubated with seminal plasma obtained from normozoospermic donors and males

Table 2 Statistical comparison of high and low molecular weight DNA fragments found in the seminal plasma of males with SCI (S) and normozoospermic donors (N).

Group	<i>n</i>	SW (<i>p</i> value)	Mean (Median)	95% CI (L–U)	Test statistic
SLMW	12	0.907 (0.196)	165.2 (166) bp	158–171	$t = -3.204/p = 0.004$
NLMW	12	0.994 (0.422)	195.5 (190.5) bp	175–215	
SHMW	12	0.982 (0.989)	3481 (3533) bp	3206–7356	$Z = 1.021/p = 0.249$
NHMW	12	0.731 (0.002)*	4004 (3744) bp	3445–4563	

SLMW SCI LMW, NLMW normozoospermic LMW, SHMW SCI HMW, NHMW normozoospermic HMW, SW Sharpio-Wilk test for normality, L lower boundary of 95% CI, H Upper boundary of 95% CI.

*Statistically significant.

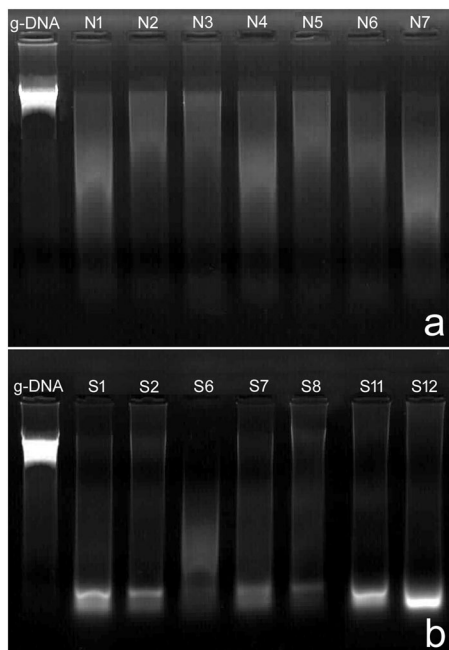


Fig. 3 DNase activity after digestion of genomic DNA with seminal plasma (a) DNase activity in normozoospermic donors (N) and (b) DNase activity in spinal cord injured males (S). Genomic DNA (g-DNA) incubated with dH₂O (control) is indicated as the first line in each panel.

with SCI. DNase activity in the normozoospermic donors was present producing a variable smear in different individuals (Fig. 3a). In terms of whole genome digestion, males with SCI (Fig. 3b) exhibited an equivalent level of DNA digestion to that observed in normozoospermic individuals. The prominent DNA bands identified as cfDNA in males with SCI were not digested as a result of the DNase activity present in these same individuals (Fig. 3b).

To analyze if the low molecular weight DNA bands observed in the males with SCI were end products of the digestion of the purified whole genomic DNA by seminal plasma nucleases, DNase digestion of the genomic DNA was tested to assess if this activity could be inhibited by the cation divalent chelator EDTA or the Ca⁺⁺ specific chelator EGTA. DNase activity was inhibited by EDTA but not by EGTA (Supplementary Fig. S1), indicating that seminal plasma nuclease activity in the males with SCI was independent of the presence of Ca⁺⁺. Inhibition of DNase

activity had no effect on the appearance of low molecular DNA bands, indicating that these bands were not the result of the digestion of the genomic DNA substrate.

DNase I digestion of purified cfDNA and plasma cfDNA

The existence of elevated levels of cfDNA in the seminal plasma of males with SCI in the presence of DNase activity could possibly indicate that the cfDNA was not accessible to the action of seminal plasma DNase. To test this hypothesis, isolated cfDNA and raw total seminal plasma were digested with recombinant DNase I at increasing concentrations and incubation times. While purified cfDNA was almost completely digested with 1 IU of DNase I, cfDNA in raw seminal plasma was digested with 5 IU (Supplementary Fig. S2a). Furthermore, purified cfDNA was completely digested with 1 IU of DNase I after 5 min, while cfDNA in raw seminal plasma remained undigested until 15 min of incubation (Supplementary Fig. S2b).

In situ hybridization of cfDNA

A DNA probe prepared from isolated cfDNA and directly labelled with tetramethyl-rhodamine-5-dUTP was hybridized directly onto spermatozoa of three males with SCI that had an elevated level of SDF (S5, 8 and 11). Protein depletion produced prior to DNA denaturation gave rise to expanded halos of dispersed DNA. Using the cfDNA probe, positive hybridization was observed covering the halos of dispersed chromatin in all semen samples. Figure 4 presents an original visualization of the green channel (counterstain, Fig. 4b), hybridized DNA probe in red (Fig. 4c) and both images merged (Fig. 4d) and shows how the hybridized signal covered the entire available DNA.

Discussion

Males with SCI in this study displayed the permanent presence of cfDNA in their seminal plasma that was not apparent in the normozoospermic donors. One DNA band of ~150 bp that was observed in all males with SCI and

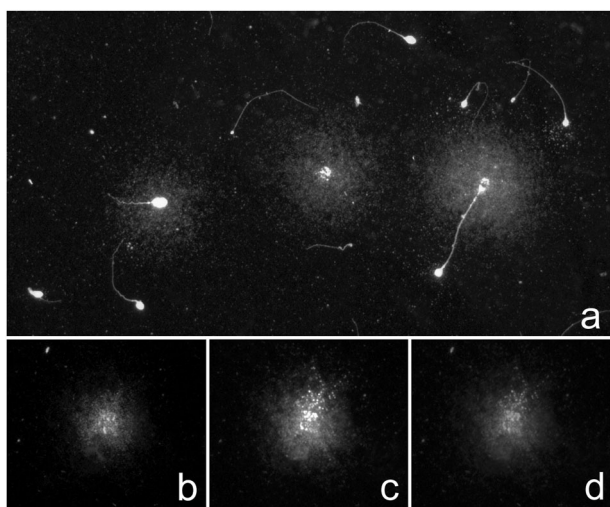


Fig. 4 Sperm DNA breakage detection—fluorescence in situ hybridization using a DNA probe prepared with circulating free DNA (cfDNA) from spinal cord injured males. (a) General view of descondensed spermatozoa after cfDNA probe hybridization (red label) and counterstained with SYBR Green (green label). (b–d) Selected spermatozoa showing background levels of staining (b: green), hybridization signal (c: red) and merged fluorescence (d).

another band of ~90 bp was present in 73% of males with SCI. The 90 bp band was only detected using standard electrophoresis gel and not detected using the bioanalyzer; this was presumably because the later technique only visualizes DNA bands formed by a double stranded DNA configuration. The results of FISH, using these DNA bands as probes, revealed an intense level of hybridization distributed over all the sperm genome.

In the males with SCI the 150 and 90 bp cfDNA bands could also be visualized when cfDNA was co-incubated with seminal plasma obtained from both normozoospermic donors and males with SCI. This indicates that functional DNase activity was present in both the seminal plasma of normozoospermic donors and males with SCI, although this activity did not appear to be operating on cfDNA. In a parallel experiment to test for the resistance of these bands to be digested with increasing levels of DNase I, we demonstrated that these bands exhibited a differential resistance to DNase I concentration. The DNase present in the seminal plasma from males with SCI was dependent on the presence of divalent cations but it appeared that Ca^{2+} was not necessary for its action. Furthermore, cfDNA was detected in seminal plasma of males with SCI independently of whether the DNase activity was inhibited (by EDTA treatment) or not; this suggests seminal plasma DNase had no effect on the cfDNA in males with SCI.

The concentration of cfDNA and the size of fragments in seminal plasma have been shown to be associated with seminal characteristics and male genital tract pathologies. For example, higher levels of cfDNA of around 1 kb have

been positively correlated with progressive sperm motility, rapid sperm progression, normal morphology and capacitation index [19]. Other studies have found that cfDNA levels are higher in azoospermic compared to normozoospermic individuals [20]. In males affected by prostate cancer the levels of cfDNA has been found to be significantly higher than in age-matched individuals without prostate cancer [21]. Although all the males with SCI included in this study presented with high levels of SDF (>50%), the concentration of cfDNA detected did not appear to be correlated with the level of SDF, at least within the range of SDF used in this study. Further studies are required to determine whether this lack of relationship holds true for males with SCI presenting with lower levels (<50%) of SDF.

Males with SCI can be considered as extreme examples of where sperm exhibit very poor characteristics. In this experiment, we selected a group of males with SCI that additionally presented with levels of SDF greater than 50%, a level of SDF that is quite unusual, even in non-SCI individuals presenting to a fertility clinic for treatment [17]. Irrespective of the method used for quantification in the current study (spectrophotometry or fluorimetry), we found the iterative presence of LMW cfDNA fragments in males with SCI was significantly higher than in normozoospermic donors. We propose that the high cfDNA levels found in the seminal plasma of males with SCI may be a consequence of the high level of sperm apoptosis and/or to the accumulation of degenerating spermatozoa and cellular debris secreted by the accessory glands associated with anejaculation that is ultimately mixed in the seminal plasma during electroejaculation or penile stimulation. Our proposal is consistent with studies that report the distal migration and storage of spermatozoa into the seminal vesicles [22] leading to reduced sperm motility [23] and the exposure of spermatozoa to abnormal changes in seminal plasma associated with seminal vesicle pathology [24]. These observations further agree with the results obtained with FISH, where the hybridization signal appeared to map all over the nuclear DNA. If this is the case, the DNA band that was used as a probe must be associated to equivalent or even identical genome domains that would be present in every chromosome.

It has been known for some years that mature spermatozoa can bind and internalize exogenous DNA [25, 26]. Although in vitro studies have demonstrated that internalization of low concentrations of foreign DNA can induce activation of endogenous nucleases that can degrade the exogenous DNA [9], a high concentration of foreign DNA may subsequently further invoke a burst of endogenous nuclease activity resulting in a partial degradation of sperm chromosomal DNA. Scenarios like this may generate new DNA fragments that are further released into the surrounding medium [9]. If spermatozoa can internalize cfDNA from the seminal plasma, then this might induce activation of endogenous nucleases

that could degrade this foreign DNA in normozoospermic samples. However, for males with SCI, the higher cfDNA concentration may not only degrade the foreign DNA but also lead to the further degradation of sperm DNA, setting up a progressively increasing vortex of DNA damage. In the context of long periods of anejaculation, this process would ultimately result in a “DNA degradation cascade”, which may account for the extremely high SDF index ($\approx 100\%$) found in some men with SCI [14].

Although contradictory results have been published regarding the relationship between cfDNA levels and DNase activity [27, 28], our study appears to be the first in which both parameters have been examined together in seminal plasma. While males with SCI in our study presented higher levels of cfDNA than normozoospermic donors, no appreciable differences were found in seminal plasma DNase activity. This finding suggests that DNase activity is not implicated in the appearance of cfDNA. On the other hand, we have demonstrated that purified cfDNA is readily digested with recombinant DNase I but cfDNA in raw seminal plasma is more resistant to this degradation. This finding suggests that in the seminal plasma, cfDNA may circulate in vesicles that protect these molecules from the action of seminal plasma nucleases. To some extent, this response of cfDNA would be similar to that found in the exosomes where a form of membrane protection exists [29, 30]. The possibility of a partially protected DNA molecule is also congruent with the finding of a differential DNA digestion to DNase, as reported in our results.

With respect to the clinical application of our findings, we have demonstrated an interesting positive relationship between cfDNA and SDF such that it might be possible to use the presence of the 150 bp cfDNA band as biomarker for evaluating high SDF. This biomarker might then be subsequently used to evaluate different protocols or therapies designed to reduce SDF. Before we go down this path, we first need to examine the equivalent marker in patients with lower levels of SDF. Secondly, if the 150 bp band is present, then we might use its presence after performing various sperm selection procedures or in the evaluation of therapies designed to reduce SDF in SCI men, such as systemic antibiotic, anti-inflammatory and antioxidant cocktails. Although the presence of high levels of cfDNA in SCI patients appears to be associated with elevated levels of SDF, further analysis is necessary to confirm the actual origin of these cfDNA fragments, whether the presence and quantity of these fragments is correlated with SDF, and whether this relationship is causal.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Acknowledgement This paper is dedicated to the fond memory of the late Prof. Roberto Mezzanote (1943–2020); a life devoted to friendship and science.

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Author contributions EVB was in charge of patient involvement and contributed to the experimental design and provided cases for inclusion of the study and editing of initial drafts of the manuscript. JV-B was instrumental in conducting the molecular biology and along with JG, CL-F, J-LF and SJ was instrumental in the experimental design, statistical analysis, composition and draft and final writing and editing of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study was supervised and approved by the clinical ethic committee of the National Hospital of Paraplegics (Toledo, Spain). We further certify that all applicable institutional and governmental regulations concerning the ethical use of human were followed during the course of this investigation.

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