SPERM DNA INTEGRITY AND MALE INFERTILITY

EXPLORING THE ROLE OF DNA FRAGMENTATION AND CHROMATIN PACKAGING

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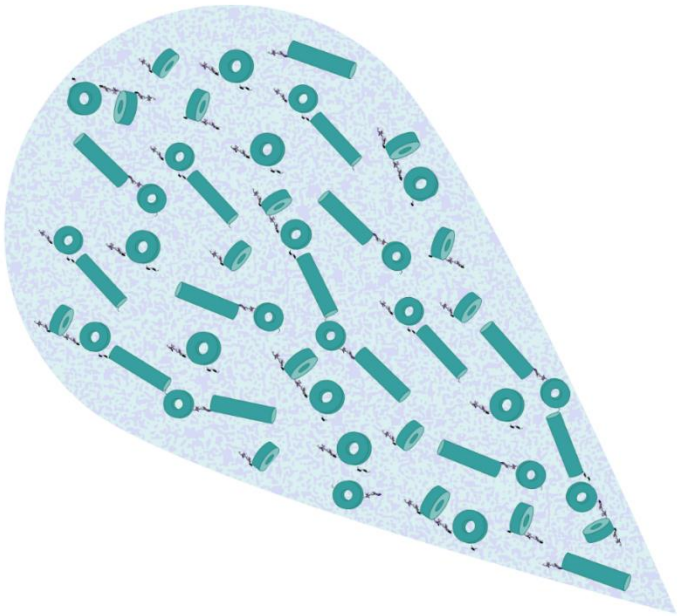

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ABSTRACT

The current evaluation of the infertile male is largely focused on the assessment of alterations in sperm count, motility, and morphology. However, sperm DNA fragmentation (SDF) has recently emerged as a potential diagnostic tool for male infertility, as high levels of SDF have been associated with a decreased fertility. The relationship between SDF and oxidative stress in semen and the function of protamines in DNA packaging to protect paternal DNA from damage are still not fully understood and require further research. In this study, we investigated the association between different types of SDF, seminal parameters, oxidative stress, and alterations in sperm chromatin compaction. SDF was measured using Comet assay, and the samples were stratified based on the presence of high levels of double-stranded DNA breaks, single-stranded DNA breaks, or both. Additionally, we measured seminal parameters using optical microscopy, oxidative stress through measuring the redox potential using the MiOXSYS system, and the protamine content by extraction and acid protein electrophoresis. We also set up novel methodologies to deeper evaluate sperm chromatin state in presence of SDF, as an adapted Western blot procedure to detect phosphorylation of basic proteins and a protocol to specifically isolate and sequence fragmented DNA. Our results showed a positive association between increased levels of sperm oxidative stress and the number of cells with both types of SDF. Of note, patients with high percentage of cells with SDF in conjunction with altered Protamine 1/Protamine 2 ratio showed a significantly increased quantity of DNA with double strand breaks per cell. Therefore, this study not only sheds light on the relationship between sperm DNA fragmentation and chromatin packaging, but also provides valuable insights and tools into the SDF knowledge and evaluation.

INTRODUCTION

MALE INFERTILITY

Infertility is understood as a disease defined by the impossibility of achieving a successful pregnancy after 1 year or more of regular unprotected intercourse [1–3]. According to the World Health Organization, infertility is a global health issue affecting 48 million couples, and 186 million people in reproductive age will seek reproduction care [1,4]. Nowadays, approximately 2.2% of the children born in Europe are conceived through Assisted Reproductive Technologies (ART) (5). Recent studies have proposed that an increase in infertility could be caused by environmental or lifestyle factors [5–7].

Overall, the male factor substantially contributes to approximately 50% of all infertility cases [2]. Currently, the evaluation of male infertility is limited to the assessment of seminogram, which can reveal gross deficiencies in sperm count, motility, or morphology, thus resolving obvious cases of male infertility [8]. Identification of infertility causes in patients with abnormal seminal parameters is not possible in approximately 30% of the cases, understood as idiopathic infertility [1,2,8,9]. Additionally, 25% of infertile men present normal seminogram termed unexplained infertility [1,2,8,9]. Knowledge of the unknown causes of infertility would help to create better diagnoses, and thus optimize and specialize treatments to increase the success rate of assisted reproductive techniques (ART).

Beyond basic sperm parameters, other molecular factors such as both sperm DNA and chromatin integrities might have a detrimental impact into normal fertilization, embryo development, and success of ART [10]. Therefore, there is a growing need to develop more complete evaluations of male factor beyond the seminogram, thus obtaining greater knowledge of male fertility that could explain ART failures.

SPERM CHROMATIN

Sperm cells are highly specialized delivery vehicles for paternal chromatin cargo composed of DNA and its associated proteins. Sperm chromatin compaction is part of the complex process of male germ cell development known as spermatogenesis. Spermatogenesis is divided into three well-defined phases: (1) a mitotic phase, during which type A replicating spermatogonia maintain the germ cell niche, while type B spermatogonia commit to meiosis to generate primary spermatocytes, (2) a meiotic phase, during which primary spermatocytes undergo two consecutive meiotic divisions, the first generating two diploid secondary spermatocytes, and the second without a previous DNA replication step generating haploid round spermatids, and finally (3) a differentiation phase called spermiogenesis, during which post meiotic round spermatids undergo marked nuclear, cytoplasmic, and morphological changes to elongated spermatids [11].

During spermiogenesis, human sperm chromatin undergoes a marked remodeling, in which histones are sequentially replaced by specific histone variants, transition proteins, and, finally, protamines through a process known as the nucleo-histone to nucleo-protamine (NH-NP) transition. This process results in a unique chromatin structure where protamines tightly pack 85-95% of the DNA and the remaining 5-15% of DNA continues attached to histones [Figure 1] [11,12].

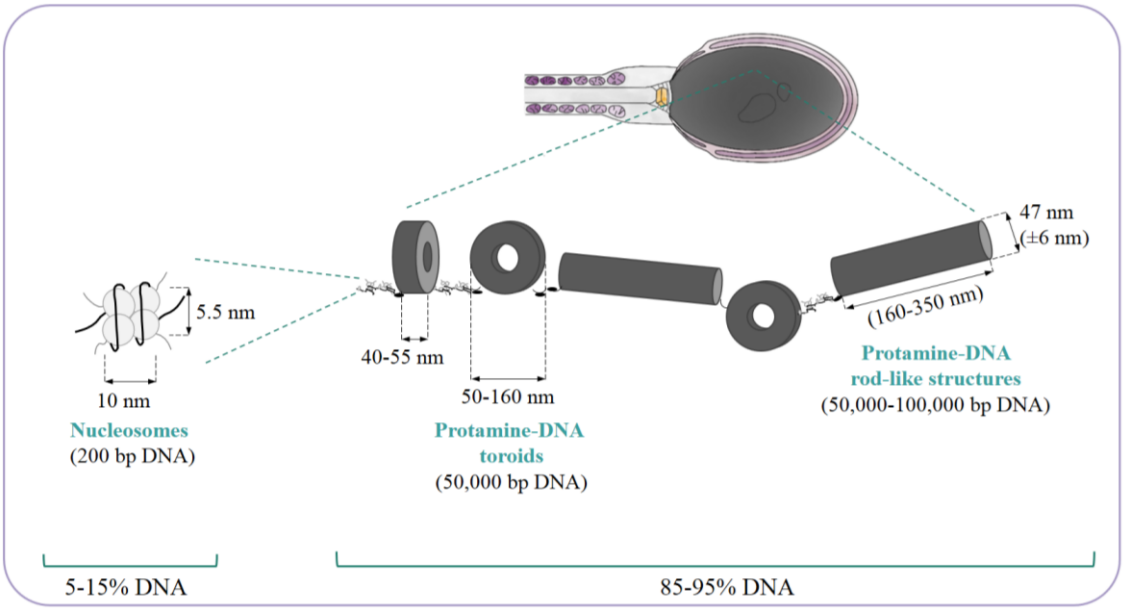

Figure 1. Chromatin structure of mature spermatozoa. The highly condensed structure is accomplished by the association of DNA with protamines that replace histones during spermiogenesis. This process results in a more tightly packed chromatin structure that forms toroidal and rod-like structures. *Adapted from de la Iglesia et al, 2023.*

Protamines are small and extremely basic specific sperm proteins (with 50-70 % of arginines residues in their sequence) where the particular amino acid composition leads these proteins to be able to compact 250 times more DNA than a nucleosome, forming highly condensed toroidal and rod-like structures [Figure 1] [11,13]. This highly compact chromatin structure is essential for the protection of the paternal genetic message from nucleases and contributes to obtaining the required hydrodynamic shape for mature sperm functionality [14].

In humans, there are two types of protamines, protamine 1 (P1) and protamine 2 family (P2) encoded by a single copy of protamine 1 (*PRM1*) and protamine 2 (*PRM2*) genes. Interestingly, P1 is translated as a mature protein of 51 amino acid residues, whereas P2 is translated as an immature protein of 102 amino acid residues (P2 precursor; pre-P2) that after proteolytic processing generates the mature forms of P2 family (HP2, HP3, and

HP4), comprising residues 46−102 (HP2), 49−102 (HP3), and 45−102 (HP4) of pre-P2, differing among them only in the 1−4 amino acid residues from the N-terminal end [Figure 2] [15,16]. HP2 is the most abundant proteoform, followed by HP3 and HP4 [11,17].

These two types of mature protamines are usually found in a 1:1 ratio (0.8-1.2) [12,15,18]. P1/P2 ratio alterations have been correlated with altered seminal parameters, sperm DNA fragmentation (SDF), and low success rate of ART [10,19]. Furthermore, alterations in pre-P2 levels are correlated with lower fertility and higher SDF [11,12,15,16,20–22]. P1 and P2 knockout mice generated by CrispCas9 showed spermatozoa with abnormal morphology and high levels of DNA damage, thus suggesting a relationship between an abnormal sperm DNA compaction by protamines and DNA damage [23–25]. With this evidence, it has been proposed that sperm chromatin maturity and integrity are essential for sperm function and subsequent embryonic development. Nevertheless, the specific deregulations that results in an altered P1/P2 ratio have been not deep-rooted.

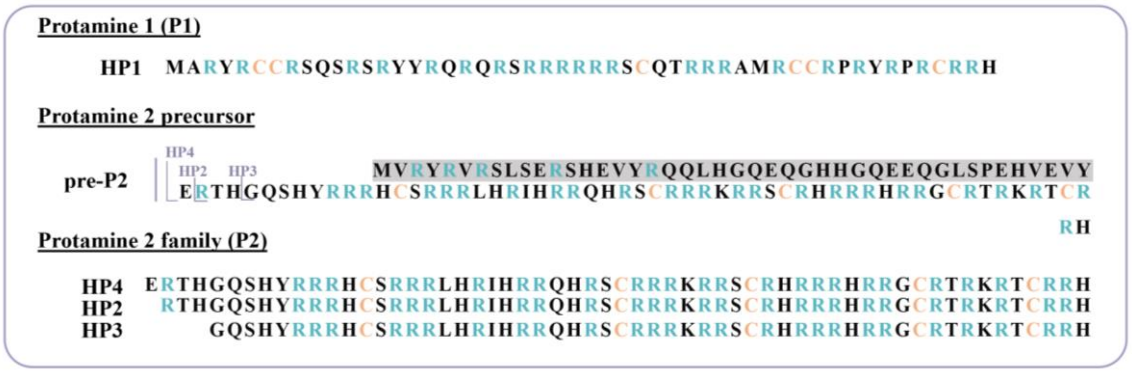

Figure 2. Protamines protein sequences. The P1, pre-P2, and P2 family are represented by their respective amino acid sequences. In the pre-P2, the proteolytic sites to generate the mature forms of the P2 family are marked.

Specific post-translational modifications (PTMs) of histone H4 such as hyperacetylation are necessary for a correct NH-NP transition process [11,17]. Recent studies have identified that PTM of protamines, specifically phosphorylation, have also a pivotal role regulating NH-NP transition process. Specifically, the replacement of histones with protamines is dependent on phosphorylation protamine status. The P1 and P2 mature proteoforms are extensively phosphorylated during NH-NP transition by SRSF protein kinase 1 (SRPK1) and calcium/calmodulin-dependent protein kinase IV (CAMK4), respectively [11,17]. Notably, CAMK4 knockout mice display spermatogenic defects and are infertile due to the specific loss of P2 [11,17]. However, once the P1 and P2 mature proteoforms are tightly bound to the DNA, an extensive dephosphorylation wave occurs before the spermatozoa enter into the epididymis, making the protamines more negatively charged and thus promoting a more tightly sperm chromatin compaction [11,17]. Interestingly, mice deficient in a heat shock protein and chaperone required for the recruitment of Serine/threonine-

protein phosphatase PP1-gamma (PP1γ), a specific phosphatase from sperm chromatin, are infertile. This is probably due to the low activity of this phosphatase, resulting in an abnormal increase in P2 phosphorylation in epididymal sperm [11,17]. This finding suggests a possible relation between increased phosphorylated proteoforms, male infertility, and mature sperm defects.

Recent studies using top-down proteomics have established a specific phosphorylation pattern of protamines [11,17]. Three different phosphorylated proteoforms for P1 (mono-, di-, triphosphorylated P1), along with unmodified P1, were identified. Additionally, pre-P2 was detected in both its unmodified form and with several PTMs, including a pattern of monophosphorylation, diphosphorylation, triphosphorylation, and tetraphosphorylation. Moreover, for the P2 mature proteoforms, one amino acid (S59) was found to be phosphorylated in both the HP2 and HP3 components, while no PTMs were identified in HP4. The unmodified HP2 was the most frequently identified mature P2 proteoform, followed by unmodified HP3, unmodified HP4, monophosphorylated HP2, and monophosphorylated HP3 [11,17]. However, the potential role of these modifications has not yet been fully addressed.

In fact, phosphorylation is known to affect how protamines interact with DNA, and given their implications with the packaging state, it can be affirmed that sperm chromatin is more complex than previously assumed. It has been proposed that the alteration of phosphorylated protamine patterns could be related to sperm DNA damage and/or altered epigenetic marks of the sperm chromatin. Therefore, research to elucidate if there are relationships between protamines PTMs and SDF is needed. Furthermore, studying protamine PTMs might explain infertility in some patients.

SPERM DNA FRAGMENTATION

DNA damage leading to SDF can occur during spermatogenesis and/or during transport through the reproductive tract, affecting one or both strands of the DNA helix and resulting in single- (SSBs) or double- (DSBs) strand DNA breaks [26,27]. Extrinsic factors such as exposure to environmental pollutants, certain chemotherapeutics, heat, smoking, and intrinsic factors like defective maturation, abortive apoptosis, and oxidative stress (OS) can cause DNA impairment [10,19]. Recent studies have shown that distinct types of breaks in sperm DNA (such as SSBs and DSBs) can result in varying effects on reproductive outcomes [10,19]. SSBs breaks can occur at multiple break points throughout the genome and are commonly associated with OS [28]. Otherwise, DSBs can result from the failure of DNA repair during meiosis and can cause an increased risk of miscarriage, lower embryo quality, and higher risks of implantation failure during ICSI cycles [28].

Therefore, understanding the pathophysiology of the different types of sperm DNA damage and their impact on male fertility is essential to provide possible screening tools, treatments, and advice for men who are facing reproductive treatments. Additionally, the evaluation of the diverse types of SSBs and DSBs can be useful in determining an

individual's predisposition to specific complications, thus improving the accuracy of diagnosis and reproductive treatment options.

Various assays are available to evaluate the integrity of sperm DNA, but nowadays the Comet assay is the best technique that can distinguish between SSBs and DSBs. Originally, the comet assay was used as a qualitative analysis, but now, with the help of specialized software such as the CometAnalyzer, it is possible to obtain quantitative data from the comet-shaped images of cells, providing more information on the amount of fragmented DNA in each cell [29]. One of the unique features of the Comet assay is that it allows for the visualization and quantification of DNA damage in the form of a comet tail, which appears when DNA fragments are pulled out of the nucleus during electrophoresis. The length of the “comet tail” indicates the extent of DNA damage, while the "comet head" contains the intact genetic material that remains in the nucleus. Therefore, the amount of DNA in the comet tail is proportional to the amount of DNA damage in the cell [Figure 3] [29].

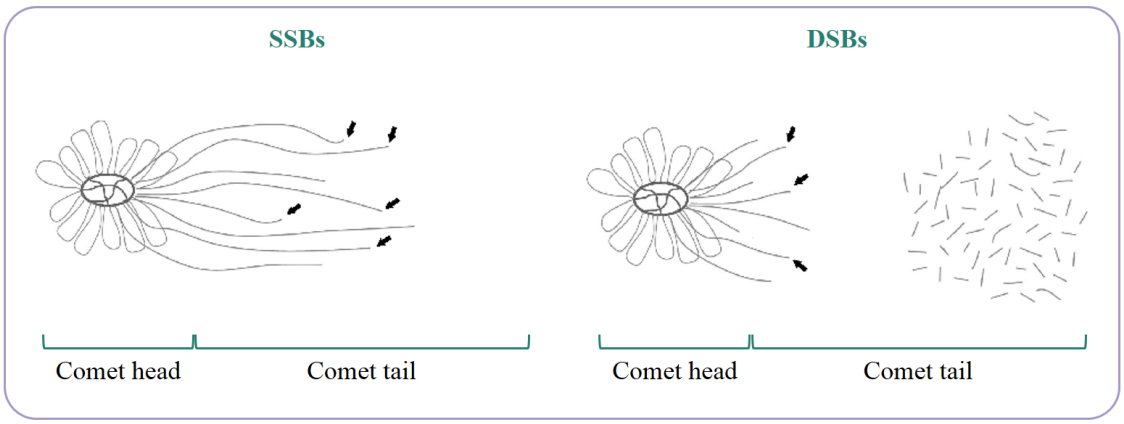

Figure 3. Graphical reproduction of two types of SDF (SSBs and DSBs) using the comet assay. Are represented the “comet head” (intact DNA) and the “comet tail” (fragmented DNA) for both types of SDF. *Adapted from Ribas-Maynou et al, 2019.*

Several studies have suggested that SDF may be related to poor chromatin compaction, which can lead to infertility or increased risk for genetic abnormalities in the offspring. Therefore, it is essential to measure the quantity of fragmented DNA in sperm using the Comet assay and evaluate if it is associated with alterations on chromatin packaging proteins such as protamines. The quantification of SDF using the Comet assay is still under development, and further studies are required to investigate the relationship between DNA damage and chromatin compaction. However, as the CometAnalyzer proves to be a useful tool for measuring DNA damage in sperm, and it can provide valuable information for clinicians to make recommendations for couples undergoing fertility treatments.

OBJECTIVES

Over the years, the assessment of sperm DNA damage in reproductive clinics has garnered increasing attention, specially the assessment of percentage of cells with DSBs. The aim of this study is to investigate the relationship between sperm DNA integrity, seminal parameters, oxidative stress, and sperm protamine compaction in males with idiopathic infertility.

We hypothesize that the quantity or quality of fragmented DNA will correlate with altered protamination, which are proteins that play a crucial role in sperm chromatin packaging. This study also aims to explore if alterations in specific PTMs of protamines such as phosphorylation, could have an impact on the percentage and/or type of DNA damage. Additionally, to enhance our knowledge about sperm DNA damage we would like to explore whether certain genes are more prone to fragmentation than others.

The specific objectives of this study could be divided in:

1. To explore the relation between single- and double-stranded sperm DNA fragmentation, seminal parameters, and oxidative stress.
2. To explore the relationship between single- and double-stranded sperm DNA fragmentation and the chromatin packaging state of spermatozoa from men with idiopathic infertility. Chromatin packaging state will be evaluated by the relative abundance of both protamines and specific PTM protamine proteoforms. Therefore, this objective will be divided in:
    - To correlate the percentage of fragmented cells and type of damage with protamine levels (P1/P2 ratio).
    - To correlate the percentage of fragmented cells and the type of damage with the state of protamine phosphorylation.
3. To explore whether some genes are more prone to fragmentation than others by sequencing fragmented DNA contained in comet tails.

MATERIAL AND METHODS

SAMPLE COLLECTION AND PROCESSING

Semen samples (n=165) were obtained with prior consent from men who attended the Andrology Unit of the Hospital Clínic de Barcelona who underwent semen analysis for fertility purposes. Ejaculates were collected after 3-5 days of sexual abstinence and a seminogram was routinely performed evaluating sperm concentration, motility, vitality, and morphology, according to the guidelines of the World Health Organization [30]. In addition, fresh semen reduction-oxidation potential (sORP) was measured by galvanostatic system MiOXSYS and normalized by concentration (sORP/C) that reflects the oxidative state of the sample. The remaining aliquots were cryopreserved using liquid nitrogen and Cryosperm® cryoprotectant in accordance with the manufacturer's instructions.

DSBs AND SSBs EVALUATION USING COMET ASSAY

Comet assay is a methodology that allows distinguishing between SSB and DSB. This assay is based on sperm inclusion in an agarose gel, followed by a nuclear decompaction, and an electrophoresis under alkaline (main detection of SSBs) or neutral (main detection of DSBs) conditions [31,32]. DNA fragments (comet tail) migrate more than the whole intact chromatin (comet head), thus forming a shape similar to a comet and being able to discriminate between positive (fragmented) and negative (non-fragmented) cells [Figure 4].

The procedure utilized for these tests is based on the protocols from Simon and Carrell (2013) and Ribas-Maynou (2012) [31,32], The process begins with the inclusion of approximately 10,000 spermatozoa from cryopreserved semen samples in a 1% agarose gel with low melting point (LMA) on a slide that has been previously treated with a thin layer of 1% agarose with a normal melting point (NMA) to prevent detachment. The use of LMA allows it to remain liquid at 37ºC and prevents damage to the cells at higher temperatures as required by NMA. Two slides are prepared for each sample: one for the alkaline comet and the other for the neutral, in addition to an extra slide for each assay that serves as a positive control. To prepare the positive control for the alkaline test, sample is previously treated with H_2O_2 500mM for 1 hour at 37ºC.

Once the slides with the gels are prepared, they are subjected to a three-phase lysis treatment of 60, 30, and 90 minutes each. During the lysis treatment, the slides are first submerged in a solution of 2.47 M NaCl, 98.9 mM EDTA, 9.8 mM Tris, and 1% Triton X-100. Then 0.5 mM DTT is added, followed by 0.2 mM SMOOTH. After lysis treatment, the gel corresponding to the positive control for the neutral test is incubated

with 0.1 units of DNase for 30 minutes at 37ºC. Before electrophoresis, all slides are immersed for 30 minutes in a solution of electrophoresis.

To conduct the electrophoresis of both comet assays, the source is set to 22V, and the mA are closely monitored to prevent overheating of the gels (<280 mA) for about ten to fifteen minutes. The use of electrophoresis solution with an alkaline pH (0.2M NaOH, 0.001M EDTA, pH 13) or neutral pH (0.05M Tris Base, 0.15M Sodium acetate trihydrate, pH 9) allows for the detection of single or double-strand breaks, respectively.

After electrophoresis, the slides with the gels undergo either a precipitation treatment using 1mM ammonium acetate in 95% ethanol (for the neutral comet assay) or a cleaning process using purified water and the Milli Q system (for the alkaline comet assay). The gels are then fixed with 70% ethanol and labeled with fluorescent DNA (SYBR Gold), allowing the fragmented nuclei to be visualized as comet tails, while unfragmented nuclei appear large and spherical [Figure 4]. Using fluorescent microscopy, the percentage of fragmented cells is quantified in the sample with respect to the total number of cells counted (from 60 to 120).

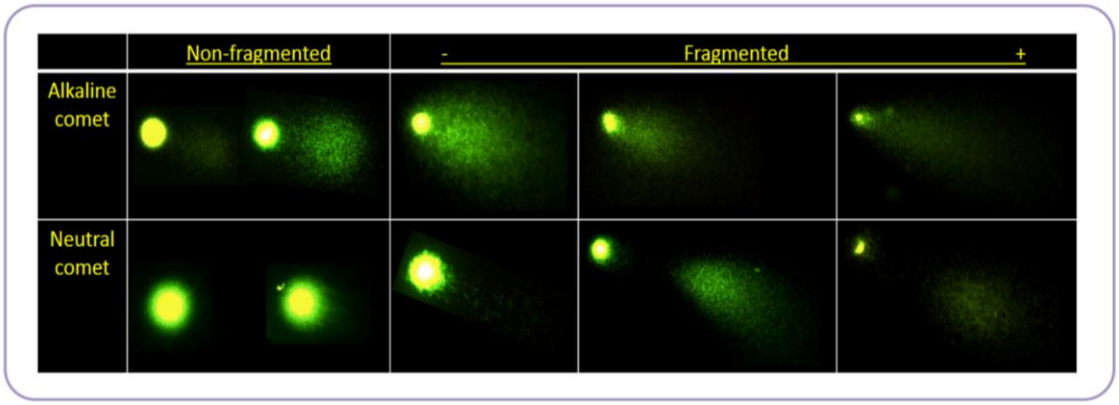

Figure 4. Results from Comet assay. Summary table of the different comet-shaped sperm cells observed in the neutral and alkaline comet assays ranked in order of increasing DNA fragmentation.

QUANTIFICATION OF FRAGMENTED DNA

To conduct a preliminary deeper analysis of the state of DNA fragmentation, the comet assay data of eleven patients with high SSBs and ten patients with high DSBs were evaluated. Between twenty and sixty cells were randomly selected from each sperm population, captured under the fluorescent microscope, and then analyzed with CometAnalyser software (v 1.0) [29]. The features “Tail Percent DNA” and “Tail Extent Moment” were used to evaluate the entity of DNA damage. “Tail Percent DNA” is a measure of the proportion of the total DNA that is present in the comet tail, while “Tail

Extent Moment" is a value that considers both the amount of DNA in the comet tail and the tail length.

SETTING UP THE NEW COMET TAIL DNA ISOLATION TECHNIQUE FOR SUBSEQUENT DNA SEQUENCING (CometTailSeq)

This technique is based on the neutral comet assay and has the purpose to isolate the fragmented double-stranded DNA (Comet tail) to be sequenced to check if specific genes are prone to be fragmented. This new technique is based on sperm localization in 1mm line in an agarose gel, and a longer electrophoresis under neutral (main detection of DSBs) conditions [31,32].

The initial step was to establish a method for localizing the sperm within a narrow zone of the gel to facilitate separation of the comet tails. Multiple attempts were made, including creating a cut with a pipette containing the sample after gelification and introducing a thread before gelification.

After successful localization of the sperm, experimentation was initiated to facilitate tail separation. A representative comet-shaped cell was measured to determine the appropriate duration for electrophoresis [Figure 5]. Based on our analysis, it was determined that a migration distance of approximately 1 cm could be achieved with an electrophoresis duration of approximately 15 hours.

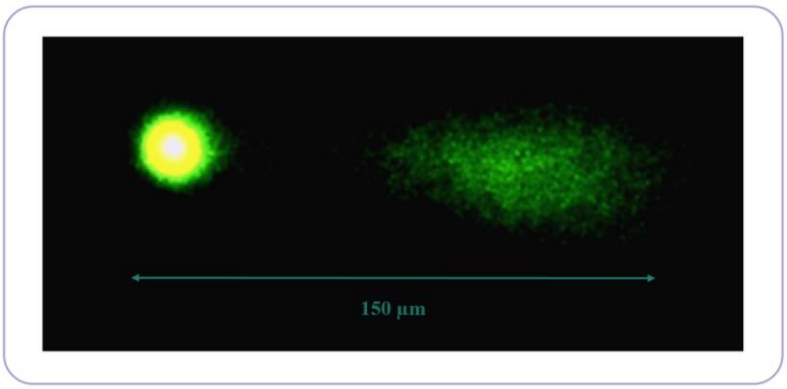

Figure 5. Representation of a comet-shaped cell that was measured to determine the optimal electrophoresis duration.

Subsequently, the DNA extraction was performed using the QIAEX® II Gel Extraction Kit in accordance with the manufacturer's instructions. The extracted DNA was quantified using the Qubit fluorometer.

PROTAMINE EXTRACTION AND QUANTIFICATION

Protamine extraction was performed following the protocols standardized by our research group [33] in some samples of each group of study (high DSBs and SSBs (n = 6), high DSBs (n = 5), high SSBs (n = 6), and low DSBs and SSBs (n = 5)) with at least 3 million spermatozoa (Mz) available after SDF evaluation by comet.

The extraction of the set of basic nuclear proteins of the sperm was conducted from cryopreserved samples, following the protocol described by Soler-Ventura et al. (2018) (29). The first step is the permeabilization of the cells with a 0.5% solution Triton X-100, 20 mM Tris pH8, and 2 mM MgCl2. The next wash is with 1mM PMSF in Mili Q water to lyse cells by osmotic shock. Then it is denatured chromatin with a solution of 20 mM EDTA, 1 mM PMSF, and 100 mM Tris-HCl at pH8 homogenizing well with the pipette and, without intermediate centrifugation, to which 6M is added guanidine hydrochloride and 575 mM DTT and mixed by shaking so that begin to see a white mucus corresponding to the sperm chromatin. The reaction is stopped with absolute ethanol and allowed to precipitate for at least 10 minutes a -20ºC. The core nuclear proteins are extracted with incubation at 37ºC with 0.5 M HCl for 5+2 minutes and are precipitated with 20% cold trichloroacetic acid. The proteins are washed with 1% β-mercaptoethanol in acetone and dried in a speed-vacuum to be finally resuspended in acid sample buffer (5.5 M urea, 20% β-mercaptoethanol, and 5% acetic acid).

Once the protamine extract was obtained, P1 and P2 were quantified by acid-urea polyacrylamide gel electrophoresis in conjunction with increasing quantities of a standard of human protamines. First, a pre-electrophoresis is performed to remove ions, and then an electrophoresis where the isolated protamines run together with purified protamine standards that will allow their quantification. Finally, the gels are stained with EZBlueTM® following the manufacturer's manual and scanned with the GS-800TM Calibrated Imaging Densitometer. The quantification of the bands is carried out by determining the optical density with the Quantity One software.

In addition, the DNA pellet obtained after protamine extraction was used for DNA quantification through 0.5 M perchloric acid hydrolysis (90°C for 20 min). The absorbance at 260 nm was determined using a NanoDrop spectrophotometer in order to normalize the amount of protamines with the amount of DNA for each sample.

DETECTION OF PHOSPHORYLATED PROTAMINES BY MODIFIED WESTERN BLOT

To detect phosphorylated proteoforms, we set up acidic western blotting protocol using purified protamine standards. We tested, different primary antibodies and blocking solution dilutions, as well as different ECL systems to visualize the western blot.

Protamines from our homemade protamine standards were separated using acid-urea polyacrylamide gel electrophoresis as previously described.

For protein transference, a polyvinylidene difluoride membrane (Immobilon®-P), four pieces of Whatman paper, and two pieces of Scotch-Brite were wetted in transferring buffer (0.9 mM Acetic acid) for at least 10 minutes. The membrane was wetted in methanol and washed with distilled water before being wetted in transferring buffer. The sandwich for the transference was then prepared in the following order: positive pole, one piece of Scotch-Brite, two pieces of Whatman paper, gel, membrane, two pieces of Whatman paper, one piece of Scotch-Brite, negative pole. The proteins were subsequently transferred to the polyvinylidene difluoride membrane (Immobilon®-P) using wet blotting for one hour at 75V.

For the blocking, we use a solution based on BSA, since the regularly used milk has casein which is a phosphoprotein and could interfere with the phosphorylation detection. To optimize the blocking step, two different BSA dilutions (3% and 5% BSA) were tested.

Following the hour-blocking step in 1XTBS-0.1% Tween 20 solution containing BSA powder, the membranes were incubated with an Anti-Phosphoserine (ab9332) primary antibody overnight at 4°C. To test this step, we diluted the antibody at 3 µg/mL, 2 µg/mL, and 1 µg/mL.

Signal detection was achieved by washing the membranes with 1XTBS-0.1%Tween 20 solution and subsequently incubating them with ECL Anti-Rabbit IgG, Horseradish Peroxidase (HRP) secondary antibodies. To optimize the detection of phosphorylation with horseradish peroxidase substrate optimized for chemiluminescence, two ECL reagents were tested (WesternBright® ECL HRP substrate and NZY Advanced ECL). Finally, the detection of the western blots was performed using the Chemidoc system.

STATISTICAL ANALYSIS

All the data were analyzed using GraphPad Prism version 7.00. A histogram graph was plotted to visualize the frequency distribution of the population (n = 165) [Figure 6]. The parameters examined included SDF, sperm concentration, sperm motility, sperm vitality, sperm morphology, sORP/c, P1/P2 ratio, and protamine (P1, P2, and P1 + P2)/DNA content of the native semen were analyzed using correlational plot, ANOVA or Kruskal-Wallis test. Post-hoc analysis was conducted using Dunn's, Holm-Sidak's, and Tukey's multiple comparison tests to identify significant differences between groups. The features obtained from CometAnalyser (tail moment and tail % DNA) were analyzed using a correlational plot and unpaired t-test with Welch’s correction or Mann-Whitney test. The data were presented with mean ± SD values in Tables 1, 2, and 3. P-value <0.05 was considered statistically significant.

FREQUENCY DISTRIBUTION OF SDF IN A POPULATION OF MEN WITH IDIOPATHIC INFERTILITY

A histogram graph was plotted to visualize the frequency distribution of SDF in our population (n = 165) [Figure 6]. The population was stratified into three categories based on the proportion of fragmented cells: high (> 36.69% SSBs; > 33.12% DSBs), medium (between 20.94-36.69% SSBs; between 21.84 – 33.12 DSBs), and low (< 20.94% SSBs; < 21.84% DSBs). The thresholds were determined via the Terciles method, according to relevant literature [31,34]. For this study, the samples were selected and stratified based on the presence of high levels of DSBs, SSBs, or both, resulting in four categories: high DSBs and SSBs (n = 32), high DSBs (n = 7), high SSBs (n = 8), and low DSBs and SSBs (n = 25).

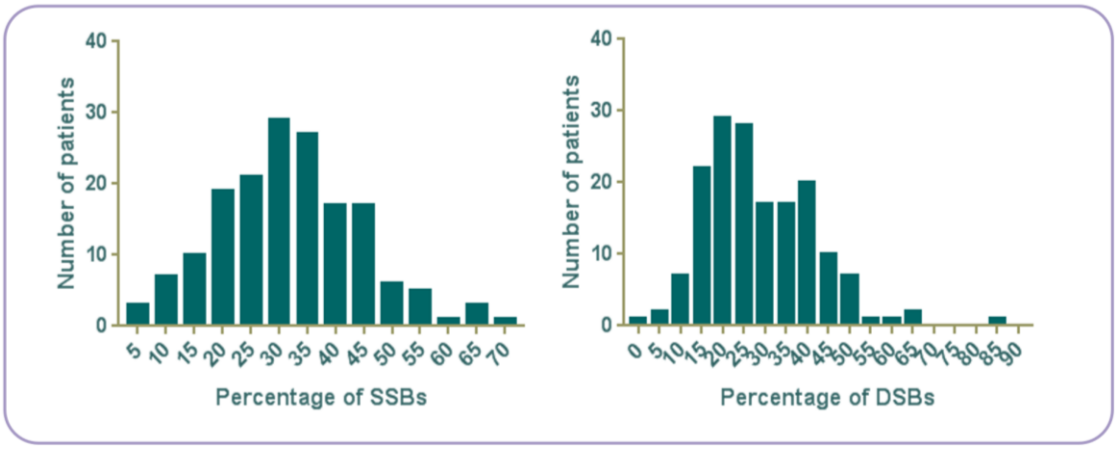

Figure 6. Graphical representation of population frequency distribution (n=165) represented as a histogram graphic according to the percentage of cells with SSBs and DSBs.

RELATIONSHIP BETWEEN DNA DAMAGE, SEMINAL PARAMETERS, AND OXIDATIVE STRESS

A first correlation approach was conducted to investigate the relationship between SDF (SSBs and DSBs), seminal parameters, and OS in the seventy-two infertile men characterized in the four study groups. The study revealed a positive correlation between sORP/C level and both DSBs (R=0.3938, P=0.0008) [Figure 7A] and SSBs (R=0.3526, P=0.0030) [Figure 7D]. Additionally, sperm concentration and vitality were negatively correlated with both DSBs (R=-0.2578, P=0.0288; R=-0.5074, P=<0.0001) [Figure 7C, Figure 7B] and SSBs levels (R=-0.2833, P=0.0159; R=-0.5535, P=<0.0001) [Figure 7F,

Figure 7E]. Furthermore, a negative correlation was observed between the proportion of cells with progressive motility and SSBs (R=-0.2060, P=0.0295) [Figure 7G].

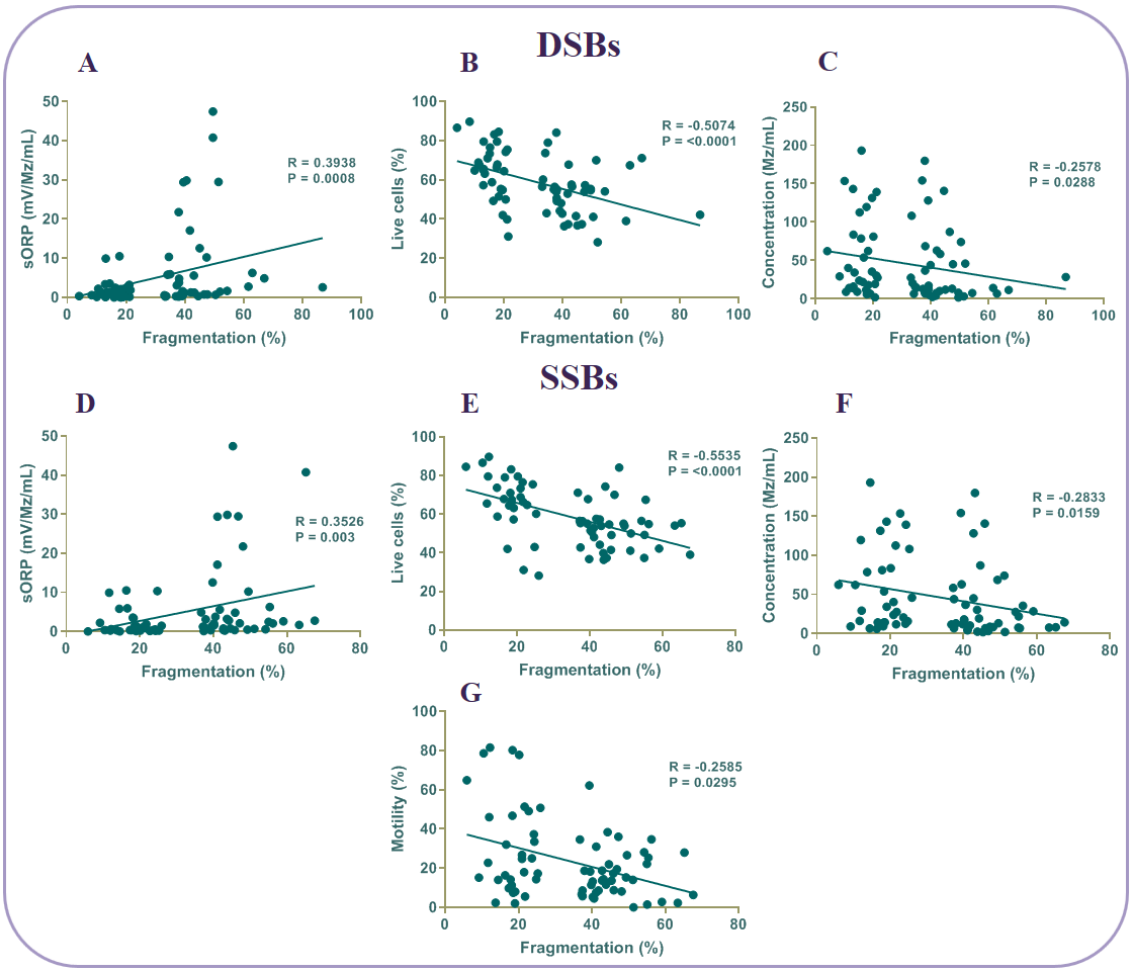

Figure 7. Graphical representation of the population distribution of semen oxidative stress (A and D) levels evaluated sORP/C, sperm concentration (C and F) vitality (B and E), and progressive motility (G) illustrating the differences observed between high levels of cells with both DSBs and SSBs versus low levels of cells with both DSBs and SSBs.

All the correlations were validated when we analyzed the differences between the 4 groups established, except for sperm motility [Table 1]. Subsequent comparison revealed significantly lower vitality (P = 0.0016) and higher sORP/C levels (P = 0.005) in samples featuring high SDF levels, for both DSBs and SSBs, compared to those characterized by low SDF [Table 1, Figure 8].

Table 1. Association between the proportion of cells with DNA fragmentation and seminal and oxidative stress-related parameters. Results showed as mean ± SD. NS: Non-significant.

	sORP/C (mV/Mz/mL)	Concentration (Mz/mL)	Progressive Motility	Immotile sperm	Morphology	Vitality
1. High fragmentation (DSBs and SSBs)	9.16 ± 13.08	40.3 ± 48.9	16.75 ± 11.95	74.86 ± 20.64	4.1 ± 20.64	53.92 ± 13.88
2. High DSBs	3.53 ± 3.915	32.03 ± 35.93	27.27 ± 13.66	68.88 ± 14.67	6.857 ± 14.67	56.82 ± 21.2
3. High SSBs	2.127 ± 0.989	17.83 ± 11.62	20.28 ± 15.62	76.88 ± 15.91	2.714 ± 15.91	53.61 ± 10.45
4. Low fragmentation (DSBs and SSBs)	1.71 ± 2.802	66.04 ± 55.00	33.1 ± 27.17	61.95 ± 28.65	5.292 ± 28.65	68.81 ± 13.55
P value (ANOVA or Kruskal-Wallis test)	0.0102	0.0430	NS	NS	NS	0.0020
P value (Dunn's and Holm-Sidak's multiple comparisons test)	0.0050 1 vs 4	NS	NS	NS	NS	0.0016 1 vs 4

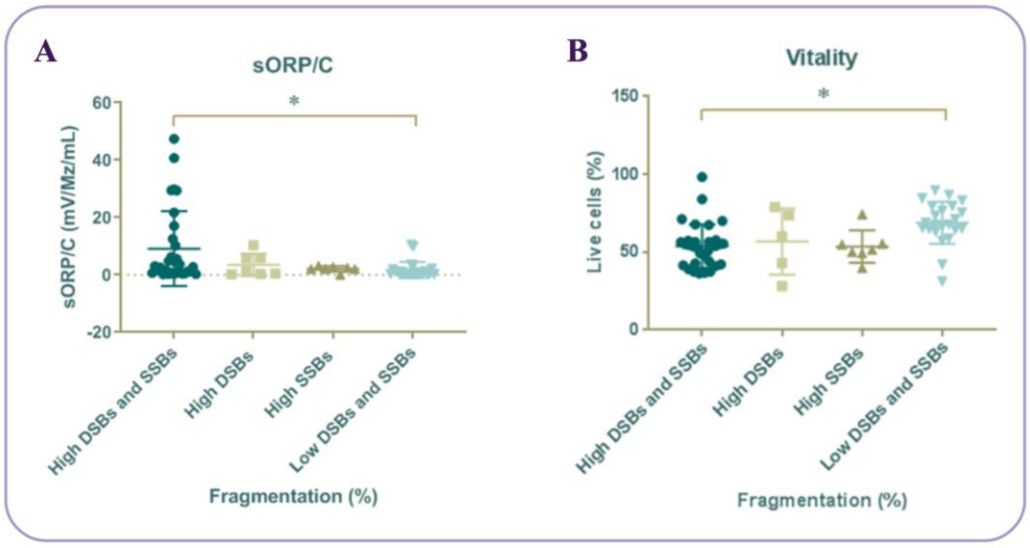

Figure 8. Graphical representation of **A)** semen oxidative stress levels evaluated sORP/C and **B)** sperm vitality illustrating the marked differences (*) observed between high levels of cells with both DSBs and SSBs versus low levels of cells with both DSBs and SSBs.

LACK OF ASSOCIATION BETWEEN DNA FRAGMENTATION AND PROTAMINES LEVELS

The analysis of the protamine data from 26 patients with high DSBs and SSBs (n = 6), high DSBs (n = 5), high SSBs (n = 7), and low DSBs and SSBs (n = 8) showed no significant differences in protamine relative levels between groups classified by the proportion of cells with fragmented DNA and type of DNA fragmentation [Table 2].

Table 2. Protamine relative levels according to the proportion of cells with fragmented DNA and type of DNA fragmentation. Results showed as mean ± SD. NS: Non-significant.

	P1/P2	P1/DNA	P2/DNA	P1+P2/DNA
High fragmentation (DSBs and SSBs)	1.18600 ± 0.30290	0.00050 ± 0.00032	0.00135 ± 0.00234	0.00097 ± 0.00072
High DSBs	1.27200 ± 0.40480	0.00021 ± 2.507e-005	0.00016 ± 5.111e-005	0.00038 ± 6.711e-005
High SSBs	1.21600 ± 0.15110	0.00010 ± 2.837e-005	8.255e-005 ± 2.514e-005	0.00018 ± 5.342e-005
Low fragmentation (DSBs and SSBs)	1.05100 ± 0.25650	0.00043 ± 0.00020	0.00041 ± 0.00017	0.00084 ± 0.00035
P value (ANOVA or Kruskal-Wallis test)	NS	NS	NS	NS

ASSOCIATION BETWEEN ALTERED PROTAMINE RATIOS AND SPECIFIC FEATURES OF DNA FRAGMENTATION

The analysis of the state of DNA fragmentation was performed by evaluating the features obtained from the software CometAnalyzer. Due to the variability of P1/P2 ratios inside the different groups classified by the amount of cells with DNA fragmentation, samples from the groups with high DSBs and SSBs (n = 6), high DSBs (n = 5), high SSBs (n = 7) were stratified based on their P1/P2 ratio into two groups: those with a normal P1/P2 ratio (0.9-1.1) and those with a high P1/P2 ratio (≥1.3). The comet assay data of eleven patients with high proportion of cells with single-stranded breaks (SSBs) and ten patients with high proportion of cells with double-stranded breaks (DSBs) were analyzed. Although no significant differences were observed in the SSBs group [Table 3], patients with a high proportion of cells with DSBs and altered P1/P2 ratio showed significantly higher values of Tail Percent DNA than those with normal P1/P2 ratio (p = 0.008) [Table 3, Figure 9A]. Furthermore, regarding the evaluation of the Tail Extent Moment parameter, which did

not show significant differences between groups [Table 3], a positive correlation was found with the P1/P2 ratio in patients with a high proportion of cells with DSBs [Figure 9B].

Table 3. Comparison of the alkaline and neutral comet image features conducted in high fragmentation and normal P1/P2 or high P1/P2 populations using the CometAnalyzer software. "Tail Percent DNA" is expressed in percentage, while "Tail Extent Moment" is expressed in arbitrary units. Results showed as mean ± SD. NS: Non-significant.

	SSBs (Alkaline comet features)		DSBs (Neutral comet features)	
	Tail Extent Moment	Tail Percent DNA	Tail Extent Moment	Tail Percent DNA
Normal P1/P2	282.500 ± 74.920	70.380 ± 10.490	297.500 ± 52.300	40.940 ± 4.675
High P1/P2	374.800 ± 83.740	77.010 ± 8.736	414.600 ± 132.500	51.110 ± 4.093
P-value (Unpaired t-test with Welch's correction or Mann-Whitney test)	NS	NS	NS	0.008

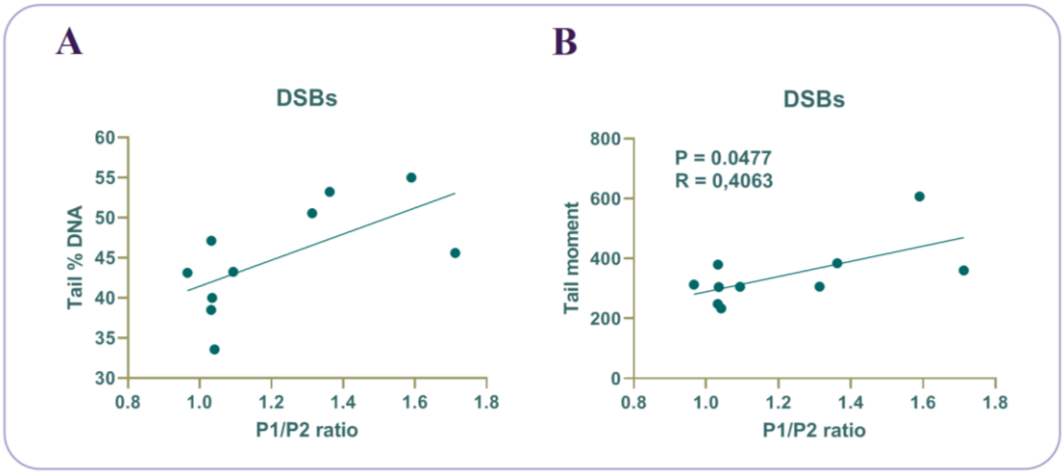

Figure 9. A) Graphical representation of Tail % DNA values in the DSBs group, illustrating the differences between patients with high and normal P1/P2 ratio. **B)** Graphical representation of Tail Extent Moment values in DSBs illustrating the correlation with P1/P2 ratio.

ACIDIC WESTERN TO DETECT PHOSPHORYLATED PROTAMINE PROTEOFORMS

We set up the acidic western to be able to detect phosphorylated basic proteins. Based on our tests, it was determined that a blocking solution containing 5% BSA produced optimal results, yielding cleaner results with low background staining. Additionally, a primary antibody dilution of 1 µg/mL allowed for the detection of more specific phosphorylated proteins [Figure 10]. Furthermore, the NZY Advanced ECL system proved capable of detecting even subtle changes that were not detectable with other systems [Figure 10 C-D]. These results enabled the observation of two adjacent clear, strong, and specific bands with lower positive charge than unmodified protamines [Figure 10D], which were not visible by EzBlue staining of the acid electrophoresis gel.

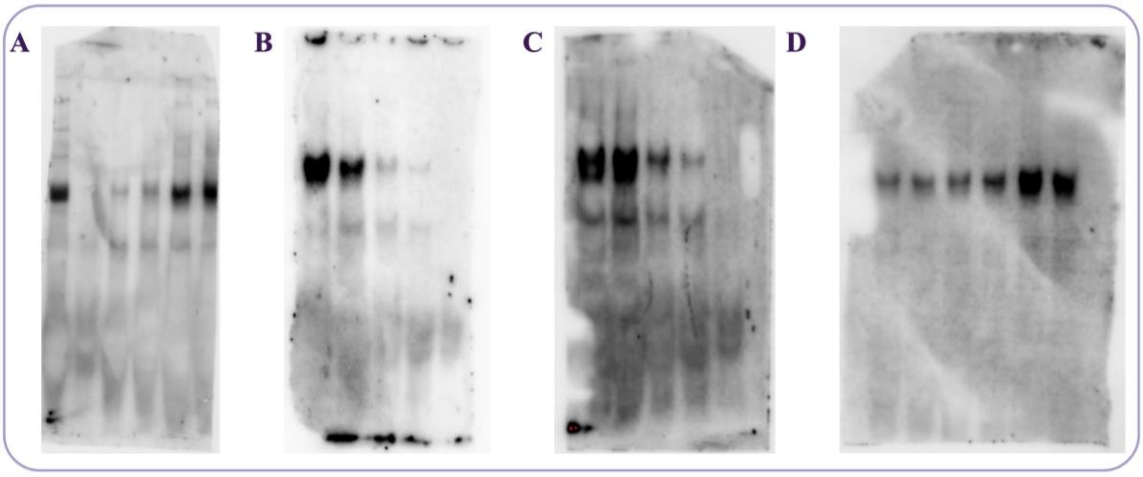

Figure 10. Western blot using different ECL systems. **A)** 3 µg/mL primary antibody, revealed with WesternBright® ECL HRP substrate. **B)** 2 µg/mL primary antibody, revealed with WesternBright® ECL HRP substrate. **C)** 2 µg/mL primary antibody, Revealed with NZY Advanced ECL. **D)** 1 µg/mL primary antibody, Revealed with NZY Advanced ECL.

COMETTAILSEQ

The sperm was successfully located within a 1mm region of an agarose gel, where it was observed that the line using the thread resulted in a cleaner and more refined loading channel [Figure 11].

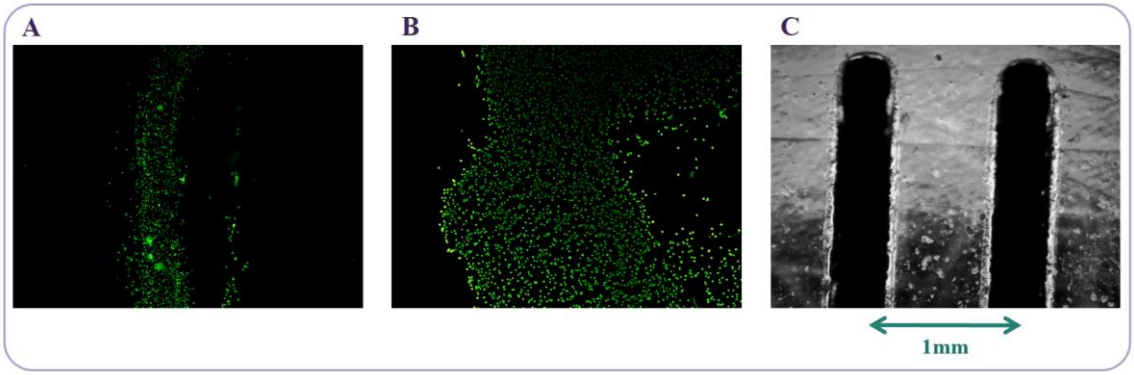

Figure 11. Location of the sample in 1 mm. A) Loading channel made with thread. **B)** Loading channel made with a pipette tip. Images A and B, have been taken by means of a fluorescence microscope at 4 magnifications. **C)** A ruler, was made with an optical microscope to give an idea of what 1 mm is.

Moreover, the tails (containing fragmented DNA) were successfully separated from the comet heads (containing intact DNA) through a 15h electrophoresis achieving a separation of approximately 3mm [Figure 12].

Figure 12. Results of the isolation of the comet tail (fragmented DNA). The image was captured with a fluorescence microscope at 10x magnification.

Finally, we isolated 7ul of DNA with a concentration of 0.322 ng/µL.

DISCUSSION

ASSOCIATION BETWEEN LOWER SPERM QUALITY, SEMEN OXIDATIVE STRESS, AND DNA FRAGMENTATION

Over the last few years, numerous studies have sought to investigate the potential relationship between sperm DNA fragmentation and conventional sperm parameters, producing inconclusive results. According to most of the literature, a negative association between SDF and sperm quality, including sperm concentration, motility, and vitality, has been observed in this study [Figure 7B, C, E-G; Table 1; Figure 8] [35–37]. Interestingly, a positive correlation between SDF and oxidative stress (OS), measured by the sORP/C parameter, has been additionally revealed, suggesting a possible association between OS and SDF. Since the presence of dead spermatozoa is known to produce reactive oxygen species (ROS), the negative association between SDF and sperm vitality makes sense [Figure 7A; D Table1; Figure 8] [38,39]. Thus, we provide novel support for the previously reported link between oxidative stress and SDF [38,39].

THE COMPLEX RELATIONSHIP BETWEEN SPERM CHROMATIN STRUCTURE AND DNA FRAGMENTATION

Based on our results, no correlation between sperm chromatin compaction, measured by the relative protamine levels, and the type of SDF, were identified, which implies that changes in P1/P2 ratios may not always result in increased levels of DNA damage and specific breaks [Table 2]. This could be attributed to the observed variability of P1/P2 ratios between patients within the same group, indicating different origins of SDF, such as defective maturation, abortive apoptosis, and OS [10,19].

However, we found a positive correlation between high P1/P2 ratios and increased double-stranded DNA breaks (Tail % DNA and Tail Extent Moment) in patients with both a high fragmentation rate and altered protamination [Table 3, Figure 9]. This implies that the occurrence of SDF is not always the same since other factors may influence this event such as chromatin protamination.

Overall, our findings suggest that aberrant chromatin compaction contributes to the occurrence of more severe double-stranded breaks, emphasizing the intricacy of chromatin structure and the higher impact of double-stranded breaks compared to single-stranded breaks [10,19].

ACIDIC WESTERN BLOT: AN IMPROVED NEW TECHNIQUE TO DETECT PHOSPHORYLATED PROTAMINE PROTEOFORMS

Using this method, we have been able to detect specific phosphorylated proteoforms of basic proteins [Figure 10D]. Based on the obtained results, a very strong band that appeared to be two distinct and adjacent bands has been observed. This suggests the presence of two different phosphorylated proteoforms with similar isoelectric points and weights. However, we have also observed other weaker bands that were not consistently present, indicating that these bands may not be highly specific. Further tests are necessary to precisely determine the nature of these findings.

Insights from unpublished results within our research group suggest that these bands may correspond to P1 proteoforms. This hypothesis is supported by prior top-down proteomic studies, which revealed greater levels of post-translational modifications on P1 than on the P2 family [17]. Nevertheless, this result should be validated, and the next step will involve identifying the specific protamine, either P1 or P2, that is phosphorylated by additional Western Blot analyses. This would enable to establish a relative quantification of phosphorylated proteoforms among patients.

It is increasingly evident that PTMs play a significant role in the interplay between chromatin structure and function [11,17]. Utilizing this novel approach, we can gain deeper insights into the complex architecture of sperm chromatin and its susceptibility to modifications such as phosphorylation. Further exploration of whether alterations in the phosphorylation of protamines are associated with SDF can shed light on the role of PTMs in the NH-NP transition, warranting additional research in this area.

COMETTAILSEQ: A NEW APPROACH FOR STUDYING DNA FRAGMENTATION

Research has increasingly acknowledged the importance of examining sperm DNA fragmentation as a critical component in the evaluation of male infertility [40,41]. The study of the genetic basis of male infertility has emerged as a key area of research, and new methods such as CometTailSeq can help to better understand the mechanisms underlying SDF.

We have demonstrated the feasibility of separating and isolating fragmented DNA [Figure 12], but due to the small size of DNA fragments, we should set up a new methodology to extract small DNA fragments from agarose gel. By identifying specific DNA fragments most affected and quantifying their size, new insights can be gained, and previously unknown aspects of SDF can be uncovered. This method can also help to determine if certain genes are more susceptible to fragmentation than others and understand their implications, thus contributing to our knowledge of the underlying mechanisms that contribute to SDF.

Overall, the application of this method offers a promising tool for further investigation and understanding of the complex relationship between DNA fragmentation and male infertility.

CONCLUSIONS

In conclusion, this study sheds light on the intricate relationship between DNA damage, seminal parameters, OS, and chromatin alterations. Our findings suggest that a lower vitality and a major presence of OS are correlated with an increase in SDF. Furthermore, our study indicates that alterations in P1/P2 ratio may not uniformly lead to increased DNA damage, but when these events occur together, patients are at a greater risk of severe double-stranded breaks.

Additionally, we present a novel tool to investigate SDF, along with an adapted tool for detecting phosphorylated protamines. These results provide valuable insights into the mechanisms of sperm DNA damage and offer potential avenues for further research to better understand the underlying mechanisms and the possible role of SDF in male infertility.

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ABBREVIATIONS

ART: Assisted Reproductive Technologies

CAMK4: calcium/calmodulin-dependent protein kinase IV

DSBs: double-strand breaks

ECL: electrochemiluminescence

ICSI: intracytoplasmic sperm injection

LMA: low melting agarose

NH-NP: nucleo-histone to nucleo-protamine

NMA: normal melting agarose

OS: oxidative stress

P1: protamine 1

P2: protamine 2 family

PP1γ: Serine/threonine-protein phosphatase PP1-gamma

Pre-P2: precursor P2

PTMs: post-translational modifications

ROS: reactive oxygen species

SDF: sperm DNA fragmentation

sORP: Static Oxidation-Reduction Potential

SRPK1: SRSF protein kinase 1

SSBs: single-strand breaks