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Sperm DNA Fragmentation in Male Infertility: Clinical Evaluation by Halo Sperm test and Protocol Development

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Dedication

*First, I thank **Allah**, the Most Merciful, for granting me the strength and patience to complete this journey.*

*To my beloved **mother**, my **sisters**, and my **brother**, thank you for your unconditional love, your constant support, and your reassuring presence through every step of this journey. Your strength, patience, and faith in me have been my greatest source of motivation. This achievement is as much yours as it is mine.*

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*To the **scientific community,** may this humble work contribute, even in a small way, to the pursuit of knowledge and future discoveries.*

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Nessrine

Dedication

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Meroua

Abstract

Infertility, defined as the inability to conceive after 12 months of regular unprotected intercourse, affects approximately 15% of couples in Algeria, representing a growing public health concern. Male factors are responsible in more than half of these cases; however, national research on male infertility remains extremely limited. This study addresses this gap by highlighting the importance of sperm DNA fragmentation testing as a key tool in the evaluation of male fertility and by proposing a cost-effective, locally applicable protocol.

Several techniques have been developed to assess sperm DNA fragmentation, including the Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, the Sperm Chromatin Structure Assay (SCSA), the Single Cell Gel Electrophoresis (COMET) assay, and the Sperm Chromatin Dispersion (SCD) test. Each offers varying sensitivity and methodological approaches to detect DNA damage in sperm cells.

The study was conducted between February and May 2025 at the IGUERLAB laboratory in Blida, Algeria, and included both a clinical and an experimental phase. In the clinical part, eleven semen samples were analyzed using the SCD method with the Halo Sperm kit to evaluate DNA fragmentation and identify contributing risk factors. These included smoking, unhealthy diet, varicocele, prolonged standing posture, exposure to cold or heat, oligo-astheno-teratozoospermia (OAT) with necrospermia, and inguinal hernias.

The results showed that all investigated factors were associated with varying levels of DNA fragmentation. For example, smokers had DFI values of 85%, 5%, and 33%; those with an unhealthy diet showed values of 25%, 85%, 40%, 93%, and 33%; and patients with varicocele presented DFI values ranging from 5% to 93%. Cold and heat exposure were also associated with elevated DFI, as were OAT and inguinal hernias, with DFI values of 25% and 72%, respectively.

In the protocol development phase, four semen samples were used across six assay rounds to refine and test a local SCD-based method. During each assay, results were compared to those of the commercial Halo Sperm kit. One assay successfully produced a clear halo, confirming the protocol's reliability. This study thus provides both clinical insights and a practical diagnostic tool to support fertility assessment in Algeria.

Key words: Sperm DNA fragmentation, Sperm Chromatin Dispersion test, Male infertility, Protocol optimization, Halo Sperm.

Résumé

L'infertilité, définie comme l'incapacité à concevoir après 12 mois de rapports sexuels réguliers non protégés, touche environ 15 % des couples en Algérie, représentant un problème croissant de santé publique. Les facteurs masculins sont responsables de plus de la moitié des cas, mais la recherche nationale sur l'infertilité masculine reste très limitée. Cette étude comble cette lacune en mettant en évidence l'importance du test de fragmentation de l'ADN spermatique dans l'évaluation de la fertilité masculine, et en proposant un protocole local économique et applicable.

Plusieurs techniques ont été développées pour évaluer la fragmentation de l'ADN spermatique, notamment le test TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling), le SCSA (Sperm Chromatin Structure Assay), le test COMET (Single Cell Gel Electrophoresis) et le test SCD (Sperm Chromatin Dispersion). Chacune de ces méthodes présente une sensibilité et une approche méthodologique différente pour détecter les dommages à l'ADN des spermatozoïdes.

L'étude a été menée entre février et mai 2025 au laboratoire IGUERLAB à Blida, Algérie, et comprenait une phase clinique et une phase expérimentale. Dans la partie clinique, onze échantillons de sperme ont été analysés selon la méthode SCD avec le kit Halo Sperm afin d'évaluer la fragmentation de l'ADN et d'identifier les facteurs de risque associés : tabagisme, alimentation déséquilibrée, varicocèle, posture debout prolongée, exposition au froid ou à la chaleur, oligo-asthéno-tératozoospermie (OAT) avec nécrospermie, et hernies inguinales.

Les résultats ont montré que tous les facteurs étudiés étaient associés à différents niveaux de fragmentation de l'ADN. L'exposition au froid, à la chaleur, l'OAT et les hernies ont également montré des DFI élevés (jusqu'à 93 %).

Dans la phase de développement du protocole, quatre échantillons ont été testés sur six essais successifs pour affiner une méthode locale basée sur le SCD. À chaque essai, les résultats ont été comparés à ceux du kit Halo Sperm. Un essai a produit un halo clair, confirmant la fiabilité du protocole. Cette étude fournit ainsi à la fois des données cliniques et un outil diagnostique local pour renforcer l'évaluation de la fertilité en Algérie.

Mots-clés: Fragmentation de l'ADN spermatique, Test de dispersion de la chromatine spermatique, Infertilité masculine, Optimisation de protocole, Halo Sperm.

الملخص

يُعرَّف العقم بأنه عدم القدرة على الإنجاب بعد مرور 12 شهرًا من الجماع المنتظم غير المحمي، ويؤثر على نحو 15٪ من الأزواج في الجزائر، مما يجعله مشكلة متزايدة في مجال الصحة العامة. العوامل الذكورية مسؤولة عن أكثر من نصف الحالات، ومع ذلك فإن الأبحاث الوطنية حول العقم عند الذكور لا تزال نادرة. تهدف هذه الدراسة إلى سد هذه الفجوة من خلال إبراز أهمية اختبار تفتت الحمض النووي في الحيوانات المنوية كأداة أساسية في تقييم خصوبة الرجل، واقتراح بروتوكول محلي منخفض التكلفة وقابل للتطبيق.

تشمل تقنيات تقييم تفتت الحمض النووي: اختبارTUNEL ، وتحليل بنية كروماتين الحيوانات المنوية(SCSA) ، واختبار الهجرة الكهربائية للخلية الواحدة(COMET) ، واختبار تشتت الكروماتين(SCD) ، وكل منها يقدم حساسية ومنهجية مختلفة للكشف عن تلف الحمض النووي.

أُجريت الدراسة بين فيفري وماي 2025 في مخبر IGUERLAB بمدينة البليدة، الجزائر، وتضمنت جزءًا سريريًا وآخر تجريبيًا. في الجزء السريري، تم تحليل 11 عينة من السائل المنوي باستخدام طريقة تشتت الكروماتين مع طقم Halo Sperm لتقييم تفتت الحمض النووي وتحديد عوامل الخطر مثل: التدخين، النظام الغذائي غير الصحي، دوالي الخصية، الوقوف لفترات طويلة، التعرض للبرد أو الحرارة، قلة وضعف حركة وتشوه الحيوانات المنوية والفتوق الإربية.

أظهرت النتائج أن جميع العوامل المدروسة ارتبطت بمستويات متفاوتة من تفتت الحمض النووي، حيث وصلت بعض القيم إلى 93%.

في المرحلة التجريبية، تم استخدام أربع عينات عبر ست تجارب متتالية لصياغة بروتوكول محلي يعتمد على مبدأ SCD. في كل تجربة، تمت مقارنة النتائج بطقم Halo Sperm. وقد أظهر أحد التجارب هالة واضحة، مما أكد موثوقية البروتوكول. تقدم هذه الدراسة بيانات سريرية وأداة تشخيص محلية تدعم تحسين تقييم العقم في الجزائر.

الكلمات المفتاحية: تجزئة الحمض النووي للحيوانات المنوية، اختبار تشتت الكروماتين، العقم الذكوري، تطوير البروتوكول، Halo Sperm.

SUMMARY

List of Figures

List of Tables

Abbreviations and Acronyms

AMAB: Assigned Male at Birth
ART: Assisted Reproductive Technology
BMI: Body Mass Index
DFI: DNA Fragmentation Index
DMRs: Differentially Methylated Regions
DNMT: DNA Methyltransferase
DSBs: Double Strand Breaks
DTT: Dithiothreitol
dUTP: Deoxyuridine Triphosphate
EDTA: Ethylenediaminetetraacetic Acid
FSH: Follicle-Stimulating Hormone
GSH: Glutathione
GnRH: Gonadotropin-Releasing Hormone
HBV: Hepatitis B Virus
HCV: Hepatitis C Virus
HIV: Human Immunodeficiency Virus
HPG axis: Hypothalamic-Pituitary-Gonadal Axis
HPV: Human Papillomavirus
HTCA: Head–Tail Coupling Apparatus
Hydroxyl (OH): Hydroxyl Radical
MAGI: Male Accessory Gland Infection
IVF: In Vitro Fertilization
LH: Luteinizing Hormone
MSV: Microsurgical Subinguinal Varicocelectomy
OAT: Oligo-Astheno-Teratozoospermia
OS: Oxidative stress
PBS: Phosphate Buffered Saline
PUFA: Polyunsaturated Fatty Acid
ROS: Reactive Oxygen Species
RTC: Randomized Controlled Trial
SARS-CoV-2: Severe Acute Respiratory Syndrome Coronavirus 2
SCD: Sperm Chromatin Dispersion
SCGE–comet: Single-Cell Gel Electrophoresis (Comet Assay)
SCSA: Sperm Chromatin Structure
SDF: Sperm DNA Fragmentation
SDS: Sodium Dodecyl Sulfate (part of buffer: SDS, 0.5M NaCl, 10mM Tris-HCl)
SFAs: Saturated Fatty Acids
SHBG: Sex Hormone-Binding Globulin
SOD: Superoxide Dismutase
SSBs: Single Strand Breaks
TdT: Terminal Deoxynucleotidyl Transferase
TFAs: Trans Fatty Acids
TNF-α: Tumor Necrosis Factor Alpha
TUNEL: Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling
WAT: White Adipose Tissue

Introduction

INTRODUCTION

Infertility is a growing public health concern, defined by the World Health Organization as the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse (Szamatowicz & Szamatowicz, 2020).

In Algeria, infertility affects approximately 15% of the population, making it a significant public health concern(Fizazi et al., 2020). With malefactors being responsible in more than half of the cases. Despite this, there is a significant lack of data and research focused on male infertility in the country. In response to this gap, a study was conducted to analyze the sperm profile in western Algeria, aiming to identify the main sperm abnormalities contributing to this widespread public health issue(Anissa Fizazi, 2022).

Male infertility is traditionally assessed by semen analysis, which measures parameters such as volume, pH, motility, morphology, and concentration. While necessary, these standard measures can be insufficient, as up to 15% of infertile men show normal semen profiles yet remain unable to conceive (McEvoy *et al.*, 2014)

This diagnostic gap has shifted attention toward sperm DNA fragmentation, which offers deeper insight into sperm quality. High levels of DNA fragmentation are linked to poor embryo development, increased miscarriage rates, and failed ART outcomes. Causes of SDF include apoptosis, protamine deficiency, and oxidative stress from reactive oxygen species in addition to environmental and lifestyle habits (Choucair *et al*., 2016) (Eisenberg *et al*., 2023) (Andrabi *et al*., 2024).

Several techniques have been developed to assess DNA integrity, including Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling, Sperm Chromatin Structure Assay, Single-Cell Gel Electrophoresis, and Sperm Chromatin Dispersion tests. Among them, the SCD method is widely used for its simplicity and effectiveness in identifying sperm DNA breaks (Ortiz *et al*., 2017) (Küçük, 2018).

In this context, the main objectives of our study were to evaluate sperm DNA fragmentation in infertile men using the SCD (Halo Sperm) test to demonstrate the clinical importance of incorporating sperm DNA testing into standard male infertility assessments. And to develop a reliable local protocol for detecting sperm DNA fragmentation adapted to the conditions of local laboratories in Algeria, especially since imported kits are often expensive and not easily accessible.

Chapter I
Bibliographic Research

I. General Concepts on Male Infertility and Sperm DNA Fragmentation

I.1 Semen and Sperm

Semen is a thick, opalescent fluid released during orgasm, containing spermatozoa and secretions from genital glands (Carroll, 2007). It provides a nourishing and protective environment with enzymes, lipids, and carbohydrates. Semen volume ranges from 0.1–10 mL, averaging 30 million sperm/mL, and varies based on ejaculation frequency (Clément, 2018). Semen includes sperm and seminal plasma, which aids in sperm transport, viability, and preparation for fertilization (Gibb *et al.*, 2021). The spermatozoon is a polarized cell with a head (haploid nucleus + acrosome), neck (HTCA), and tail (axoneme-based flagellum). The axoneme's dynein arms generate motility (Miyata *et al.*, 2024). The midpiece, rich in mitochondria, powers movement; an annulus separates it from the principal piece (Whitfield, 2024). The HTCA ensures head-tail integrity (Galletta *et al.*, 2025). The acrosome overlays the nucleus and contains cytoskeletal elements (Toshimori and Eddy, 2015). (Figure 1)

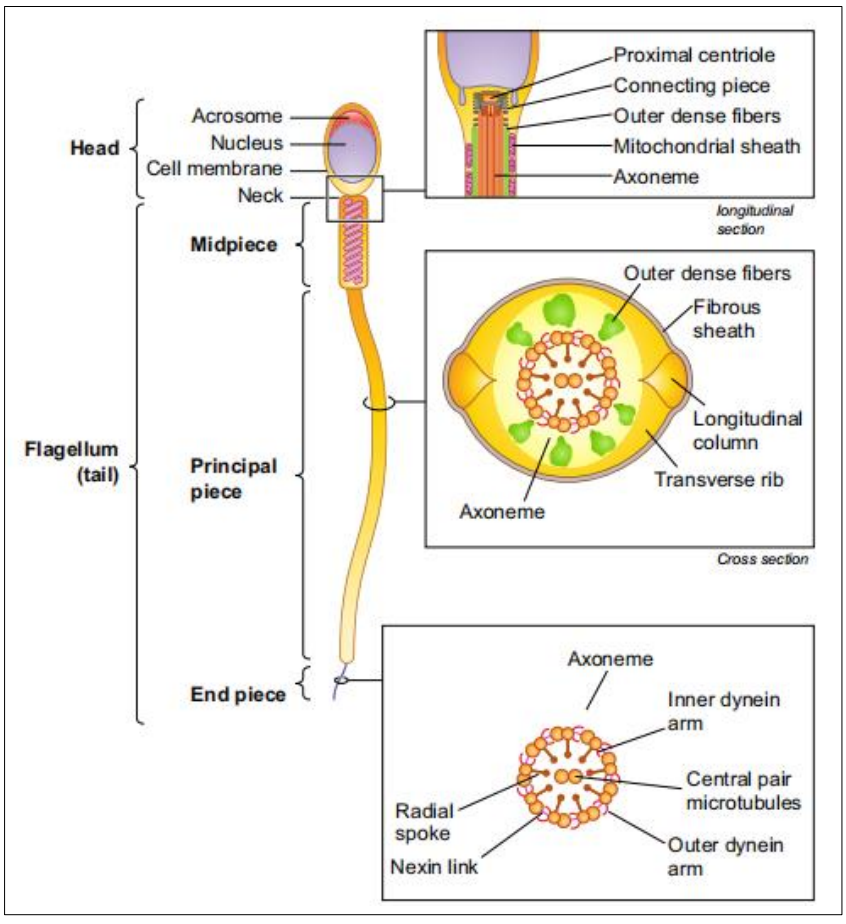

Figure 1: Morphological structure components of human spermatozoa (Teves and Roldan, 2021).

I.2 Spermatogenesis

Spermatogenesis is the process where germline stem cells in the seminiferous epithelium develop into spermatozoa (Teves and Roldan, 2022).

Usually, it is separated into three distinct phases:

- The proliferative phase is the first stage of spermatogenesis, involving the mitotic division of diploid spermatogonia. Undifferentiated spermatogonia maintain their population and produce differentiated spermatogonia, which are committed to sperm production.(Teves & Roldan, 2022)
- The proliferative phase is followed by the meiotic phase, which consists of two meiotic divisions in which primary spermatocytes reduce the number of chromosomes, resulting in genomic recombination and haploid cells (Teves & Roldan, 2022).
- Sperm differentiation depends on the final stage, spermiogenesis. The haploid round spermatids produced by meiosis experience significant morphological and functional modifications during spermiogenesis. The creation of new organelles, such as the chromatoid body made of RNA, the acrosome, which is the result of vesicle fusion, and the manchette. The spermiogenic phase is also characterized by the trafficking of proteins via the acrosome, acroplaxome, and manchette; nuclear condensation and remodeling; acquisition of the species-specific shape; removal of residual cytoplasm; flagella assembly; and spermiation (Teves and Roldan, 2022). (Figure 2)

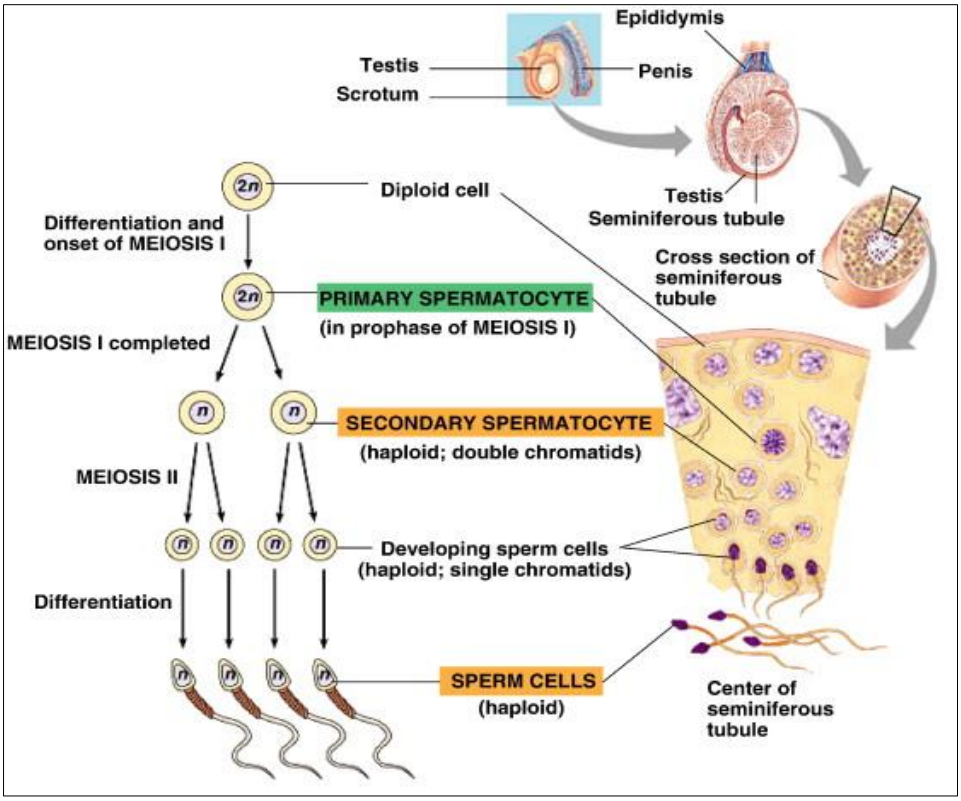

Figure 2 : A depiction of the process of spermatogenesis (Ashar *et al*., 2010).

This process is regulated by the hypothalamo-pituitary-testicular axis (McLachlan, 2000). The hypothalamus, the anterior and posterior parts of the pituitary gland and the testes make up the hypothalamic pituitary gonadal axis. Gonadotrophin releasing hormone (GnRH) is pulsatilely secreted by the hypothalamus and travels to the anterior pituitary gland via the hypophyseal portal system. This causes the anterior pituitary gland to release follicle-stimulating hormone (FSH) and luteinizing hormone (LH), two hormones essential for reproduction.

Leydig, Sertoli, and germ cells are among the testicular cells that are impacted by LH and FSH. Sertoli cells in the testicle create inhibin B, whereas Leydig cells make testosterone. The anterior pituitary and the hypothalamus get feedback from both hormones. The enzyme aromatase has the ability to change testosterone into estrogen once it enters the peripheral circulation, which may impact fertility (Clavijo and Hsiao, 2018).

I.3 Spermogram

Male fertility is assessed using a laboratory test called semen analysis. Infertility is the inability to conceive after a year of unprotected sexual activity; it affects 15% of couples who are of reproductive age. Male infertility is evaluated with a complete evaluation that includes semen assays, a comprehensive physical examination, and a detailed medical and sexual history. About half of instances of infertility have a male contributing component, and about 30% of cases have a male element (Sunder and Leslie, 2022).

The total quantity of spermatozoa, fluid volume, sperm concentration, and the spermatozoa's properties such as their motility, morphology, viability, and secretory composition are among the many factors evaluated in semen analysis (Sunder and Leslie, 2022).

A single sample may suffice if results are normal, but repeated tests are advised when abnormalities are found. Low semen volume (<1.5 mL) may suggest retrograde ejaculation, incomplete collection, or anatomical issues like ejaculatory duct obstruction or congenital absence of the vas deferens. Low sperm count can result from testicular damage due to trauma, cancer treatments, infections, or genetic conditions. Genital tract obstructions may be detected via imaging, especially in cases with normal hormone levels and testis size. Low sperm motility generally has limited impact on natural conception unless severely impaired, in which case assisted reproductive techniques may help. Poor sperm vitality may reflect issues in the epididymis or flagella defects, while abnormal morphology points to

problems in spermatogenesis. The presence of leukocytes in semen (pyospermia) may indicate genital tract infection or inflammation and should be further investigated (Sunder and Leslie, 2022).

I.4 Sperm Deoxyribonucleic Acid

DNA methylation and histone modifications critically regulate the expression of many genes and repeat regions during spermatogenesis (Liu *et al*., 2019).

During spermatogenesis, DNA undergoes passive demethylation early on, followed by new methylation by histone marks H3K4me3 and H3K36me3. Germline cells contain core histones (H2A, H2B, H3, H4) during mitosis and meiosis. In the mid-spermiogenesis elongation phase, round spermatids acquire testis-specific histone variants alongside core histones. At this stage, most histones (around 85% in humans) are replaced by protamines, resulting in a stable nucleus. It starts with testis-specific histone variants, followed by H4 hyperacetylation, temporary DNA double-strand breaks, replacement of histones with transition proteins, and finally, protamine integration. Leading to mature spermatozoa chromatin mainly composed of protamines (Marcho *et al*., 2020).

Protamines are about half the size of histones, containing 50 to 110 amino acids. They also have a higher positive charge due to their higher content of lysine and arginine, allowing them to efficiently bind to the major groove of the DNA every 10 to 15 base pairs of DNA in each double helix turn. In the mammalian genome, two types of protamines (protamine 1 and protamine 2 family) are present. Whilst the first protein is present in all mammalian sperm, protamine 2 is only found in humans, primates, mouse, rabbits and stallions to enhances condensation. The binding of protamines confers a 44-fold smaller volume of the chromatin compared to liver cells. This DNA condensation present in sperm cells leads to a strong protection of genetic content from genotoxic activity, a key feature to enable the delivery of an uninterrupted genetic information to the embryo, limiting mutations and ensuring the perpetuation of the species. However, upon fertilization, this highly condensed chromatin needs to be reestablished as a functionally active chromatin, thus requiring an interchange of protamines by histones in the male pronucleus, a process in which other proteins are also thought to be imported to paternal genome (Ribas-Maynou *et al*., 2022).

I.5. Risk factors and characteristics of sperm DNA fragmentation

I.5.1. Biological factors

I.5.1.1. Oxidative stress

Because of their high reactivity, reactive oxygen species (ROS) can harm any cell structure, including DNA molecules, but they can also have detrimental consequences by influencing cell proliferation, differentiation, and function. Seminal antioxidants effectively regulate the quantity of ROS production in the semen of fertile guys (González-Marín, Gosálvez and Roy, 2012). When ROS such as free radicals and hydrogen peroxide production exceeds the antioxidant capacity of the male reproductive system or seminal plasma, harmful repercussions result, such as DNA base modifications leading to the formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG) and lipid peroxidation. The DNA structure is destabilized by those oxidized base adducts, which results in DNA breaks (Farkouh *et al.*, 2022).

According to recent research, mature sperm may sustain DNA damage as a result of immature sperm's strong ROS production. This injury would occur following ejaculation and spermiation from the seminiferous tubules to the epididymis. By activating sperm caspases and endonucleases, ROS can either directly or indirectly harm sperm DNA. Because mature and immature sperm are in close proximity to one another under these circumstances, co-centrifugation of immature sperm (which generate high levels of ROS) with mature sperm causes sperm DNA fragmentation in mature sperm (González-Marín *et al*., 2012).

I.5.1.2. Abortive Apoptosis

Sperm DNA fragmentation (SDF) can result from abortive apoptosis during spermatogenesis, male germ cells gradually lose their ability to undergo apoptosis a type of programmed cell death a process meant to eliminate defective germ cells, as they develop into highly differentiated spermatozoa. Differentiating haploid germ cells are believed to go through a limited form of apoptosis, which results in DNA fragmentation in the nucleus, rather than a full apoptotic response that results in cell death. This process allows the cells to differentiate into mature, functional spermatozoa that may still be able to fertilize. (González-Marín *et al*., 2012).

Failure of apoptosis leads to the accumulation of spermatozoa expressing apoptotic markers in the ejaculate. Extrinsic apoptosis is triggered by Fas-ligand binding to death receptors (e.g., Fas), activating caspase-8 or -10. The presence of Fas in ejaculated sperm indicates increased abortive apoptosis. Oxidative stress (OS) can also activate apoptosis by triggering the MAPK pathway, increasing p53 and caspase-3 expression while reducing bcl-2, thus impairing maturation. Furthermore, intrinsic apoptosis is activated through mitochondrial

pathways, where cytochrome c release leads to the activation of caspases 3, 6, and 7. Phosphatidylserine externalization serves as an early marker, while SDF is a late marker of apoptosis. (Agarwal *et al*., 2020).

I.5.1.3. DNA Breaks During the Process of Spermiogenesis

DNA fragmentation can result from changes in chromatin remodelling that occur during spermiogenesis. It may be a sign of inadequate maturation during spermiogenesis if ejaculated sperm have DNA nicks. In order to relax the chromatin by histone hyper-acetylation and introduce breaks by topoisomerase II to produce and ligate nicks that promote protamination, chromatin packing may require endogenous nuclease activity. In order to facilitate chromatin arrangement during the protamines' displacement of histones, these nicks are thought to relieve torsional stress. Anomalies in the chromatin packing or unrepaired DNA nicks can occur from changes in the regulation of this process. There may be DNA fragmentation in ejaculated spermatozoa if temporary breaks are not fixed (Sakkas and Alvarez, 2010 ; González-Marín *et al*., 2012;).

I.5.2. Medical factors

I.5.2.1. Varicocele

Sperm DNA damage is linked to various disorders, including varicocele, through common pathways that increase reactive oxygen species (ROS). Varicocele is characterized by abnormally dilated veins in the spermatic cord's pampiniform plexus, which causes backward blood flow and vascular dilation. This blood reflux may result from congenital issues with venous valves. The endothelial cells in the dilated veins of the pampiniform plexus generate ROS and nitrogen species that can harm testicular and epididymal cells. Previous studies have shown elevated ROS levels in men with varicocele, along with increased oxidative stress markers like nitric oxide, malondialdehyde, hydrogen peroxide, and superoxide anion (Abdulmassih Wood *et al*., 2021).

The study of Zhang *et al*., (2022), confirms that clinical varicocele had higher DFI then healthy controls, in other words varicocele could damage sperm DNA

I.5.2.2. Genital infections

Genital tract infections significantly impair sperm quality and DNA integrity. Bacteriospermia is associated with reduced sperm motility, morphology, and concentration, and a marked increase in DNA fragmentation due to elevated reactive oxygen species (ROS) and leukocytospermia. Common pathogens include E. faecalis, E. coli, S. aureus, and S.

agalactiae, which trigger inflammation and oxidative stress, damaging sperm chromatin. (Eini *et al*., 2021).

Leukocyte infiltration (notably CD68+ macrophages and CD3+ T cells) in the testes may further disrupt spermatogenesis through cytokine production such as IL-6 and TNF-α and reduced testosterone. In COVID-19, 42% of men showed at least one abnormal sperm parameter, with DNA damage linked more to fever and inflammation than to direct viral presence (Leng *et al.*, 2023). However, Soares et Al. showed that infection with SARS-CoV-2 decreased the length of sperm telomeres (Soares *et al.*, 2025).

Viral infections like Hepatitis B Virus (HBV), Hepatitis C Virus (HCV), Human Immunodeficiency Virus (HIV), and Human Papillomavirus (HPV) can impair fertility. HBV can integrate into sperm DNA and compromise quality via DNA damage and apoptosis. HCV affects sperm motility and viability, while HIV may reduce sperm quality either directly by targeting immune cells or via antiretroviral therapy. HPV binds sperm heads and is linked to reduced motility and fertility, especially in infertile men (Garolla *et al.*, 2013).

I.5.2.3. Age

Aging in males is associated with gradual epigenetic alterations in sperm, often explained by the accumulation of random epigenetic errors (epimutations) due to prolonged time and environmental or lifestyle stressors (Ashapkin *et al.*, 2022). This results in functional decline of Sertoli and Leydig cells and impaired spermatogenesis, though the mechanisms remain partially understood. Advanced paternal age correlates with increased germline mutations including Y chromosome microdeletions, telomere elongation, oxidative stress, and DNA repair deficiencies as well as epigenetic alterations such as DNA methylation changes and altered miRNA profiles (e.g., miR-125a-5p and miR-574) which collectively impair sperm quality and fertility (Dong *et al*., 2022).

Studies show that sperm DNA methylation changes with age in specific, germline-only regions, especially near genes related to development and neural function. These epigenetic changes have been used to develop sperm epigenetic clocks, and are associated with reduced fertility and a higher risk of neurodevelopmental disorders in offspring (Jenkins *et al*., 2018).

Moreover, older men (≥40 years) are significantly more likely to have DFI >10%, even with normal semen parameters, indicating a clear link between aging and declining male fertility (Rosiak-Gill *et al*., 2019).

I.5.3. Environmental and lifestyle factors

I.5.3.1. Smoking and Alcohol

Smoking and alcohol consumption are major contributors to male infertility, primarily through oxidative stress and hormonal disruption. Smoking reduces semen volume, motility, and morphology, increases DNA fragmentation, and causes protamine deficiency, largely due to elevated ROS and increased leukocytes in semen. It also alters hormone levels which are essential for regulating spermatogenesis. These disturbances impair the body's antioxidant defense systems and negatively affect the quality of spermatogenesis, leading to lasting fertility issues and morphological anomalies in sperm cells (Amor *et al*., 2022).

Similarly, heavy alcohol intake decreases sperm count, motility, and vitality while increasing DNA damage and chromatin defects. Alcohol metabolites produce reactive intermediates like acetyl and methyl radicals which raises ROS levels and reduce antioxidants like SOD and GSH, also affecting testosterone, FSH, LH, and estradiol. These effects are dose-dependent and similar to those of tobacco, especially regarding sperm head morphology and motility (Finelli *et al*., 2021; Amor *et al*., 2022).

I.5.3.2. Diet and obesity

White adipose tissue (WAT) in obese men releases excess pro-inflammatory cytokines and reactive oxygen species (ROS), leading to systemic inflammation and oxidative stress, which compromise male fertility. WAT is also a source of aromatase, an enzyme that converts testosterone into estrogens, resulting in reduced testosterone and elevated estrogen levels. This hormonal imbalance affects the hypothalamic–pituitary–testicular (HPT) axis, causing a drop in FSH and LH secretion. The consequence is reduced stimulation of Leydig and Sertoli cells, which are essential for testosterone production and spermatogenesis. As a result, obesity is associated with altered sperm production, low sperm quality, and decreased fertility. It also secretes leptin, a hormone that is implicated in appetite regulation and stimulation of reproductive hormones; with obesity, however, leptin resistance and excess production of leptin can lead to androgen deficiency and disrupted reproductive function. Other pro-inflammatory WAT molecules, such as TNF-α, IL-6, and ghrelin, also interfere with the hypothalamic-pituitary-gonadal (HPG) axis and spermatogenesis. Obesity is also

associated with erectile dysfunction, increased scrotal temperature, and sleep apnea, which are harmful to sperm production and hormone balance (Skoracka *et al.*, 2020).

Diet plays a key role in hormonal balance. High glucose intake and insulin resistance reduce testosterone levels, while processed foods rich in xenoestrogens, saturated fats, and trans fats disturb hormonal homeostasis and reduce sperm quality. Low omega-3 intake worsens these effects (Salas-Huetos *et al.*, 2017; Kljajic *et al.*, 2021; Corsetti, Notari and Montano, 2023). (Figure 3)

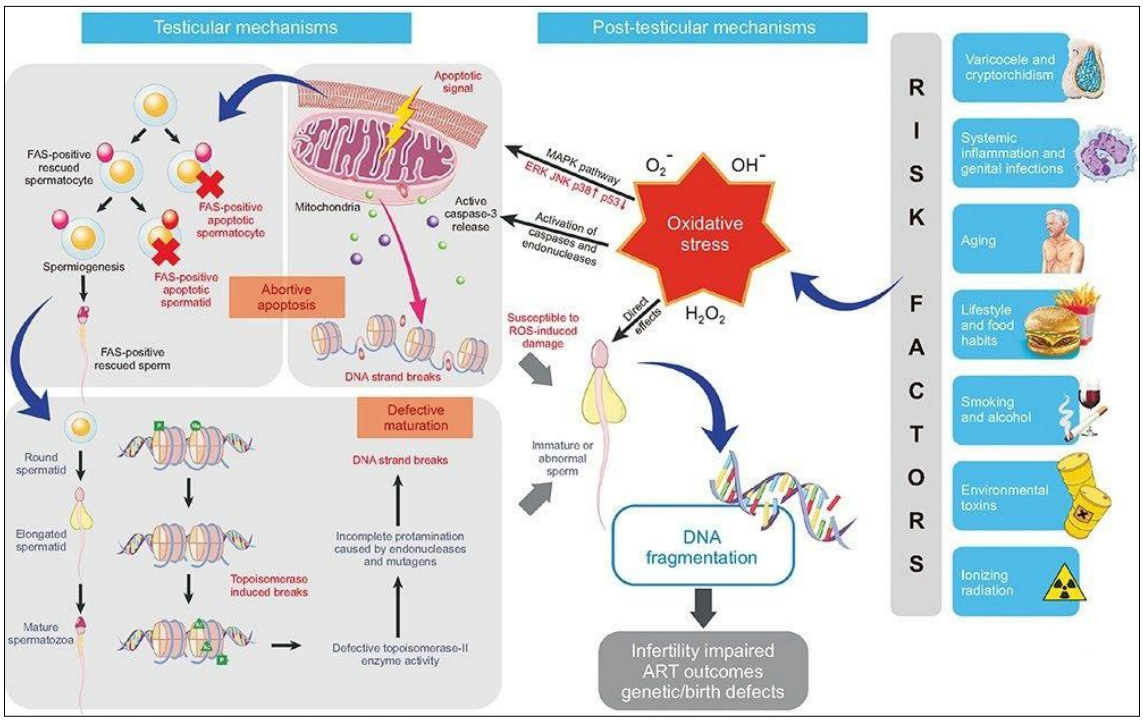

Figure 3: Overview of the Origins of Sperm DNA Fragmentation (Agarwal *et al.*, 2020)**.**

I.6. Techniques for detecting sperm DNA fragmentation

Traditional semen analysis assesses sperm count, motility, and morphology but fails to detect DNA damage. The DNA fragmentation index (DFI) measures the percentage of sperm with broken DNA and is typically higher in infertile men. Elevated DFI can impair fertilization, embryonic development, and lead to miscarriage by transmitting defective genetic material. Two meta-analyses have shown that high DFI correlates with reduced clinical pregnancy rates, lower embryo quality, and increased miscarriage risk, supporting its use as a predictive marker of male fertility (Al Omrani *et al.*, 2018; Yang *et al.*, 2024).

The most widely used methodologies for assessing sperm DNA fragmentation are (TUNEL) assay labelling via fluorescence microscopy, and flow cytometry, the sperm

chromatin dispersion assay (SCD), the sperm chromatin structure assay (SCSA), the single-cell gel electrophoresis assay (SCGE–comet) (Chatzimeletiou *et al*., 2023).

I.6.1. Terminal deoxynucleotidyl transferase dUTP nick end labeling

The TUNEL assay is one of the most common methods used to assess sperm DNA fragmentation (Takeda *et al*., 2015).

TUNEL utilizes a template-independent DNA polymerase called Terminal Deoxynucleotidyl Transferase (TdT) which non-preferentially adds deoxyribonucleotides to 3' hydroxyl (OH) single- and double-stranded DNA. Deoxyuridine triphosphate (dUTP) is the substrate that is added by the TdT enzyme to the free 3'-OH break-ends of DNA. The added dUTP can be directly labeled and therefore acts as a direct marker of DNA breaks, or the signal can be amplified by the use of a modified dUTP to which labeled anti-dUTP antibody can be adsorbed. The more DNA strand break sites present, the more label is incorporated within a cell (Sharma *et al*., 2013).

TUNEL staining has the ability to detect sperm cells with fragmented DNA in smears using fluorescence microscopy (Arifulin *et al*., 2017).

It can also be detected, by flow cytometry which allows a rapid analysis of a large number of cells in a short period of time and provides a highly sensitive, precise and objective tool to define spermatozoa with fragmented DNA (Ragosta *et al*., 2024). (Figure 4)

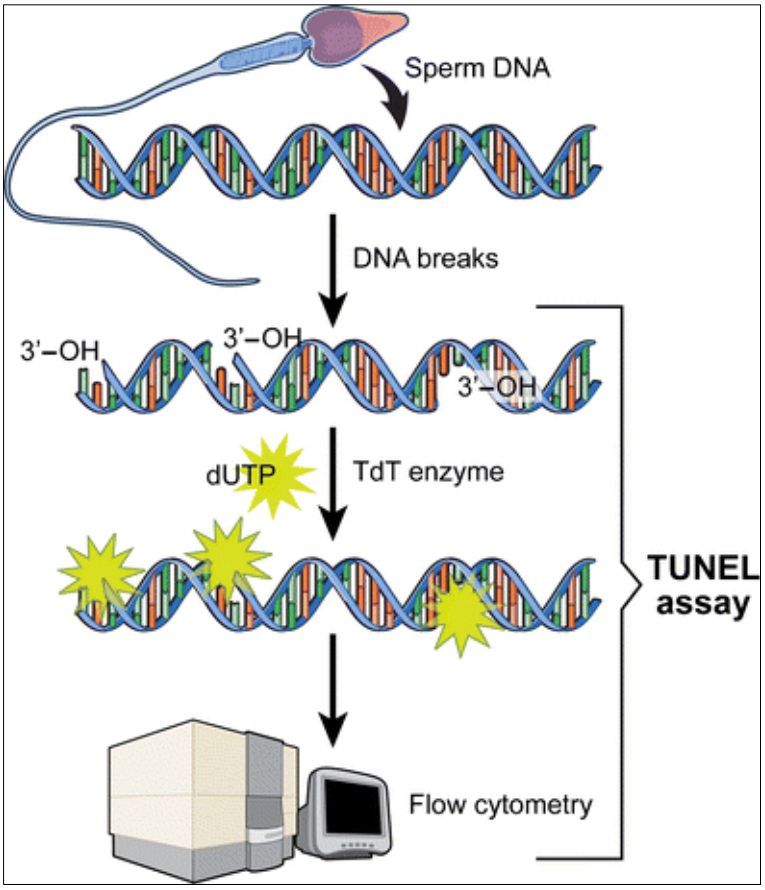

Figure 4: Schematic Representation of the TUNEL Assay (Sharma *et al*., 2016)**.**

I.6.2. Sperm Chromatin Dispersion Assay

The SCD assay can demonstrate the presence of DNA fragmentation in spermatozoa. It involves a simple, yet cost-effective procedure and gives reliable results. The inclusion of SCD test along with the regular semen analysis is likely to augment information regarding sperm fertilizing capacity (Pratap *et al*., 2017).

In SCD, sperm suspensions are embedded in agarose gel on slides and treated with an acid denaturation solution to generate restricted single-strand DNA motifs at the sites of existing single- or double-strand breaks. The denaturation is stopped, and spermatozoa are exposed to a lysing solution to remove the sperm membrane and nuclear proteins. Lastly, the slides are stained, and the percentages of sperm with non-dispersed and dispersed chromatin loops are manually assessed under fluorescence or bright-field microscopy. The halos correspond to open DNA loops attached to the residual nuclear structure, as seen in sperm with low or no SDF. By contrast, sperm exhibiting SDF show minimal or no halos (Esteves *et al*., 2022).

Sperm cells with intact DNA appear with a visible halo around the nucleus, while fragmented DNA lacks this halo. The percentage of sperm without halos represents the DNA fragmentation index: less than 15% indicates low fragmentation and good sperm quality, 15% to 30% reflects moderate fragmentation, and values above 30% signify high DNA fragmentation (Wang *et al*., 2014). (Figure 5)

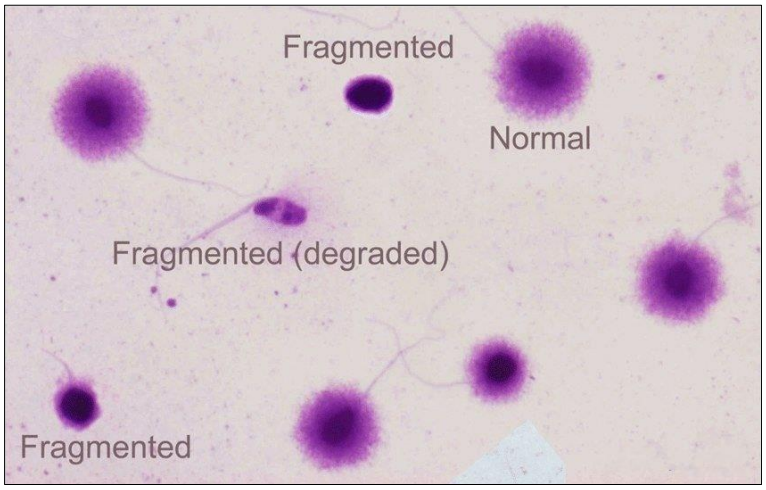

Figure 5: SCD Test: Halo patterns (Martínez *et al.*, 2018).

I.6.3. Sperm Chromatin Structure Assay

The SCSA uses acridine orange fluorescence to detect DSBs and SSBs and measures the metachromatic shift of acridine orange from green (indicating intercalation into double-stranded DNA) to red fluorescence (indicating association with single-stranded DNA) to determine the degree of cellular DNA denaturation(González-Marín *et al*., 2012). The advantages of this technique are that it's a standardized protocol and a fast assay, while the disadvantages are the need for flow cytometry and trained personnel (Adler *et al*., 2023). (Figure 6)

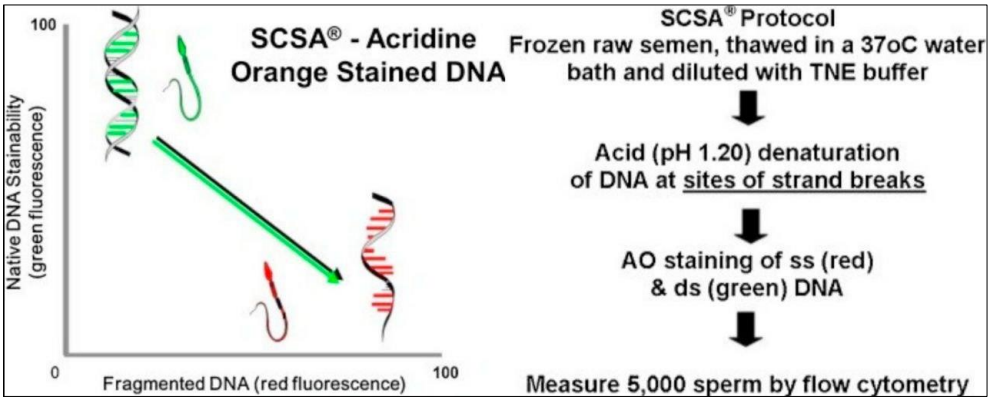

Figure 6: Schematic Representation of the SCSA Assay (Evenson, 2016).

I.6.4. Single-Cell Gel Electrophoresis (Comet Assay)

Also known as the single-cell gel electrophoresis (SCGE) assay. In the Comet assay, cells are lysed in an alkaline or neutral solution to create deproteinized nuclei and the resultant DNA is then electrophoresed. DNA fragments travel in the direction of the anode, forming a comet-like pattern. The degree of DNA damage is indicated by the length of the tail. The advantages of this technique include that it's fast assay and requires low sperm counts, while the disadvantages are the lack of standardization in technique and analysis, necessitates skill and inter-observer variability (Adler *et al*., 2023). (Figure 7)

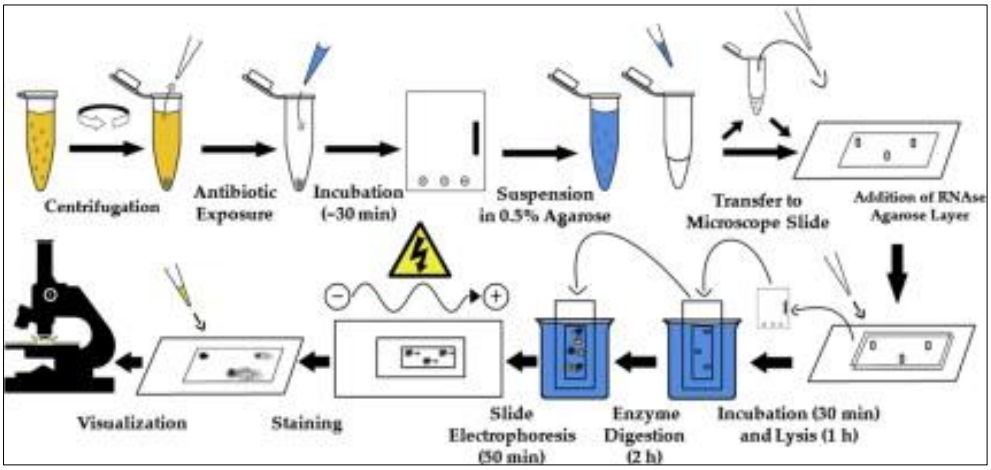

Figure 7: Schematic Representation of the COMET Assay (Solanky *et al*. 2012)**.**

Chapter II

Material and Methods

This is a prospective descriptive study that aimed both to develop a standardized protocol for assessing sperm DNA fragmentation. The primary objective of this study was to design and refine a reliable protocol for detecting DNA fragmentation and to highlight the clinical importance of this test in male fertility evaluation.

In the experimental portion of the study, four semen samples were used in a series of technical trials aimed at optimizing the protocol. In parallel, a clinical analysis of 11 patient samples was carried out to evaluate potential biological or lifestyle factors linked to DNA damage and to look into the significance of sperm DNA fragmentation testing in relation to male fertility.

The study was carried out at IGUERLAB Laboratory, located in Diar El Bahri, Beni Mered, Blida, over a period from February, 2025 to May, 2025.

II.1. Materials

II.1.1. Studied sample

Our study was conducted using 4 semen samples to test the developed protocol and compare it to a commercial kit (Halo Sperm). Additionally, 11 patients' records were reviewed to highlight the importance of the test.

II.1.2. Equipment and Reagents

The list of equipment and reagents used in the study is presented in Table III (in Appendix), along with the commercial Halo Sperm kit used for comparison.

II.2. Methods

II.2.1. Pre-analytical Phase

Semen samples were collected and handled under proper conditions to preserve integrity. Clinical data were recorded for each of the 11 patients, including age, medical and fertility history, and relevant factors (Table IV and V in Appendix). 4 semen samples were used for developing and testing the protocol under controlled lab conditions.

II.2.2. Analytical Phase

The sperm DNA fragmentation test was performed using the SCD method.

In the experimental phase, different protocols were tested on 4 samples to optimize acid concentration, lysis buffer, timing, and staining steps.

II.2.3. Halo Sperm Kit Protocol

This Halo Sperm Kit is a ready-to-use commercial system for assessing sperm DNA fragmentation. It includes pre-melted agarose (ACS), Eppendorf tubes, and four reagents

(DA, LS, SSA, SSB) for acid denaturation, lysis, and staining. The protocol uses small sample volumes, short incubation times, and operates at room temperature. The protocol consists of the following steps:

1. Melt Agarose Cell Support (ACS): Use a water bath (95–100°C for 5 min) or microwave oven (with caution). Avoid boiling. After melting, maintain at 37°C for 5 min. Tubes with melted agarose should be kept at 37°C until use.
2. Prepare sperm dilution: Dilute sperm in extender or PBS to max. 20 million/mL.
3. Mix sperm with agarose: Transfer 50 µL of sperm to Eppendorf tube with 100 µL of agarose. Mix gently without bubbles.
4. Drop sample on slide: Place 8 µL of cell suspension in the center of well “S”. Cover with coverslip. Keep slide horizontal. Prepare a control sample in well "C".
5. Solidify agarose: Place slide at 2–8°C (cold surface or fridge) for 5 minutes to solidify the agarose.
6. Remove coverslip: Gently slide it off. Keep slide at room temp (22–25°C) to dry.
7. Acid denaturation: Apply Solution 1 (DA). Incubate 7 minutes. Remove by tilting.
8. Lysis: Apply Solution 2 (LS). Incubate 20 minutes. Remove by tilting.
9. Wash: Wash slide for 5 min with distilled water.
10. Dehydrate: Dehydrate using: 70% ethanol (2 min) then 90% ethanol (2 min) then 100% ethanol (2 min). Allow to dry. Slides can be stored dry.
11. Staining: Apply Solution 3 (SSA). Incubate 7 min. Remove by tilting. Then apply Solution 4 (SSB). Incubate 7 min, remove by tilting. Air-dry.
12. Microscopy: Visualize under brightfield. Adjust staining intensity if needed by re-washing or re-staining.
13. Analysis: Count fragmented vs. non-fragmented sperm. Use formula: SDF (%) = (fragmented + degraded / total counted) × 100

Table I: Summary of Experimental Protocols for Sperm DNA Fragmentation Assays

<table>
<tr><th></th><th>Sample Embedding</th><th>Cell Lysis</th><th>Acid Treatment</th><th>Neutralization and Fixation</th><th>Staining</th></tr>
<tr><td colspan="6">Primary Protocol</td></tr>
<tr><td>First 4 slides</td><td>• Mix 50 µL of sperm sample with 100 µL of agarose (1% at 37°C).
• Place a drop on a clean slide and gently place the coverslip on the drop.
• Allow to solidify at 4°C for 5 minutes.
• Remove the coverslip and let it dry for 5min.</td><td rowspan="2">Immerse the slide in lysis buffer (1% SDS, 0.5M NaCl, 10mM Tris-HCl) for 10 minutes at room temperature.</td><td rowspan="2">Immerse the slide in 0.08M HCl for 5 minutes at room temperature.</td><td rowspan="2">• Rinse with PBS (pH 7.4) for 1 minute.
• Immediately immerse in 100% ethanol for 1 minute.</td><td rowspan="2">• Immerse in eosin Y for 5 minutes, then rinse with distilled water.
• Immerse in methylene blue for 5 minutes, then rinse and allow to dry.</td></tr>
<tr><td>Another 4 slides</td><td>• Replace agarose with nutrient agar.</td></tr>
<tr><td colspan="6">Second assay</td></tr>
<tr><td>1st slide</td><td colspan="2">One side using:
• Agarose + Sperm
• Then we continue using the commercial kit Halosperm (1,2,3,4 reagents)</td><td colspan="3">In the other side:
• Using the full kit Halosperm</td></tr>
<tr><td>2nd slide</td><td>• Mix 50 µL of sperm sample with 100 µL of agarose (1% at 37°C).
• Place a drop on a clean slide and gently place the coverslip on the drop.
• Allow to solidify at 4°C for 5</td><td colspan="4"></td></tr>
</table>

<table>
<tr><td></td><td colspan="2">minutes.
• Remove the coverslip and let it dry for 5min.</td><td colspan="4"></td></tr>
<tr><td>3rd slide</td><td>Mix 50 µL of sperm sample with 100 µL of agarose (1% at 37°C).</td><td rowspan="2">• Place a drop on a clean slide and gently place the coverslip on the drop.
• Allow to solidify at 4°C for 5 minutes.
• Remove the coverslip and let it dry for 15min.</td><td rowspan="2">Immerse the slide in lysis buffer (1% SDS, 0.5M NaCl, 10mM Tris-HCl) for 10 minutes at room temperature.</td><td rowspan="2">Immerse the slide in 0.08M HCl for 5 minutes at room temperature.</td><td rowspan="2">• Rinse with PBS (pH 7.4) for 1 minute.
• Immediately immerse in 100% ethanol for 1 minute.</td><td rowspan="2">• Immerse in eosin Y for 5 minutes, then rinse with distilled water.
• Immerse in methylene blue for 5 minutes, then rinse and allow to dry.</td></tr>
<tr><td>4th slide</td><td>• Dilution: Mix 50 µL of sperm sample with 100 µL of agarose and 110 µL of distilled water (1% at 37°C).</td></tr>
<tr><td colspan="6">Third assay</td></tr>
<tr><td>1st slide</td><td colspan="2" rowspan="3">• Mix 50 µL of sperm sample with 100 µL of agarose (1% at 37°C).
• Place a drop on a clean slide and gently place the coverslip on the drop.
• Allow to solidify at 4°C for 5 minutes.
• Remove the coverslip and let it dry for 15min.</td><td>Use the solution 1 of kit Halosperm and leave it for 10 minutes.</td><td rowspan="3">Immerse the slide in 0.08M HCl for 5 minutes at room temperature.</td><td rowspan="3">• Immerse the slide in distilled water for 5 minutes.
• Immerse in 70% ethanol for 2 minutes.
• Immerse in 100% ethanol for 2 minutes.</td><td rowspan="3">• Immerse in eosin Y (1/2 dilution) for 5 minutes, then rinse with distilled water.
• Immerse in methylene blue (1/3 dilution) for 5 minutes, then rinse and allow to dry.</td></tr>
<tr><td>2nd slide</td><td rowspan="2">Immerse the slide in lysis buffer (1% SDS, 0.5M NaCl, 10mM Tris-HCl) for 10 minutes at room temperature and let the slide dry for few minutes.</td></tr>
<tr><td>3rd slide</td></tr>
<tr><td colspan="6">Fourth assay</td></tr>
</table>

<table>
<tr><td>1st slide</td><td rowspan="2">• Mix 50 µL of sperm sample with 100 µL of agarose (1% at 37°C).
• Place a drop on a clean slide and gently place the coverslip on the drop.
• Allow to solidify at 4°C for 5 minutes.
• Remove the coverslip and let it dry for 15min.</td><td rowspan="2">Immerse the slide in lysis buffer (1% SDS, 0.5M NaCl, 10mM Tris-HCl) for 10 minutes at room temperature.</td><td rowspan="2">Immerse the slide in 0.1M HCl for 5 minutes at room temperature.</td><td rowspan="2">• Immerse the slide in distilled water for 5 minutes.
• Immerse in 70% ethanol for 2 minutes.
• Immerse in 100% ethanol for 2 minutes.</td><td rowspan="2">• Immerse in eosin Y (1/2 dilution) for 5 minutes, then rinse with distilled water.
• Immerse in methylene blue (1/3 dilution) for 5 minutes, then rinse and allow to dry.</td></tr>
<tr><td>2nd slide</td></tr>
<tr><td colspan="6">Fifth assay</td></tr>
<tr><td>1st slide</td><td rowspan="3">• Mix 50 µL of sperm sample with 100 µL of agarose (1% at 37°C).
• Place a drop on a clean slide and gently place the coverslip on the drop.
• Allow to solidify at 4°C for 5 minutes.
• Remove the coverslip and let it dry for 15min.</td><td>Immerse the slide in lysis buffer (1% SDS, 0.5M NaCl, 10mM Tris-HCl) for 15 minutes at room temperature.</td><td>Immerse the slide in 0.1M HCl for 5 minutes at room temperature.</td><td rowspan="3">• Immerse the slide in distilled water for 5 minutes.
• Immerse in 70% ethanol for 2 minutes.
• Immerse in 100% ethanol for 2 minutes.</td><td rowspan="3">• Immerse in eosin Y (1/2 dilution) for 5 minutes, then rinse with distilled water.
• Immerse in methylene blue (1/3 dilution) for 5 minutes, then rinse and allow to dry.</td></tr>
<tr><td>2nd slide</td><td>Immerse the slide in lysis buffer (1% SDS, 0.5M NaCl, 10mM Tris-HCl) for 10 minutes at room temperature.</td><td rowspan="2">Immerse the slide in 0.1M HCl for 20 minutes at room temperature.</td></tr>
<tr><td>3rd slide</td><td>Immerse the slide in lysis buffer (1% SDS, 0.5M NaCl, 10mM Tris-HCl) for 15 minutes at room temperature.</td></tr>
</table>

<table>
<tr><td colspan="7">Fifth assay - Part 2</td></tr>
<tr><td></td><td>Sample Embedding</td><td>Acid Treatment</td><td>Cell Lysis</td><td>Neutralization and Fixation</td><td>Staining</td></tr>
<tr><td>4th slide</td><td rowspan="2">• Mix 50 µL of sperm sample with 100 µL of agarose (1% at 37°C).
• Place a drop on a clean slide and gently place the coverslip on the drop.
• Allow to solidify at 4°C for 5 minutes.
• Remove the coverslip.
• Let it dry for 15min.</td><td>Immerse the slide in 0.1M HCl for 5 minutes at room temperature.</td><td>Immerse the slide in lysis buffer (1% SDS, 0.5M NaCl, 10mM Tris-HCl) for 20 minutes at room temperature.</td><td rowspan="2">• Immerse the slide in distilled water for 5 minutes.
• Immerse in 70% ethanol for 2 minutes.
• Immerse in 100% ethanol for 2 minutes.</td><td rowspan="2">• Immerse in eosin Y (1/2 dilution) for 5 minutes, then rinse with distilled water.
• Immerse in methylene blue (1/3 dilution) for 5 minutes, then rinse and allow to dry.</td></tr>
<tr><td>5th slide</td><td>Immerse the slide in 0.1M HCl for 20 minutes at room temperature.</td><td>Immerse the slide in lysis buffer (1% SDS, 0.5M NaCl, 10mM Tris-HCl) for 15 minutes at room temperature.</td></tr>
<tr><td colspan="7">Sixth assay</td></tr>
<tr><td></td><td>Sample Embedding</td><td>Acid Treatment</td><td>Cell Lysis</td><td>Acid Treatment</td><td>Neutralization and Fixation</td><td>Staining</td></tr>
<tr><td>1st slide</td><td rowspan="2">• Mix 50 µL of sperm sample with 100 µL of agarose (1% at 37°C).
• Place a drop on a clean slide and</td><td>Immerse the slide in 0.1M HCl for 5 minutes at room temperature.</td><td>Immerse the slide in lysis buffer (1% SDS, 0.5M NaCl, 10mM Tris-HCl) for 15 minutes at room temperature.</td><td>Immerse the slide in 0.1M HCl for 15 minutes at room temperature.</td><td rowspan="2">• Immerse the slide in distilled water for 5 minutes.
• Immerse in 70% ethanol</td><td rowspan="2">• Immerse in eosin Y (1/2 dilution) for 5 minutes, then rinse with distilled water.
• Immerse in</td></tr>
<tr><td>2nd slide</td><td>Immerse the slide</td><td>Immerse the slide</td><td>Immerse the slide</td></tr>
</table>

	gently place the coverslip on the drop. • Allow to solidify at 4°C for 5 minutes. • Remove the coverslip. • Let it dry for 15min.	in 0.1M HCl for 15 minutes at room temperature.	in lysis buffer (1% SDS, 0.5M NaCl, 10mM Tris-HCl) for 15 minutes at room temperature.	in 0.1M HCl for 5 minutes at room temperature.	for 2 minutes. • Immerse in 100% ethanol for 2 minutes.	methylene blue (1/3 dilution) for 5 minutes, then rinse and allow to dry.

In the first assay, the base protocol was established by mixing 50 µL of sperm sample with 100 µL of 1% agarose at 37°C. A drop of this mixture was placed on a clean slide, gently covered with a coverslip, solidified at 4°C for 5 minutes, and then the coverslip was removed before air-drying. In additional slides, nutrient agar was used instead of agarose to evaluate its effect on embedding.

The second assay focused on comparative testing with the Halo Sperm commercial kit. On the first slide, one half was prepared using agarose with sperm followed by treatment with the Halo Sperm kit reagents (1 to 4), while the other half used the full kit procedure. The second slide followed the basic agarose protocol only. The third slide was fully processed using our in-house protocol. In the fourth slide, the agarose was diluted by adding 110 µL of distilled water to the sperm-agarose mix to test its impact on dispersion.

In the third assay, we evaluated whether our lysis buffer affected halo formation. The sperm was embedded in agarose, and the lysis step was replaced with reagent 1 from the Halo Sperm kit. Neutralization and fixation were modified using 5 minutes in distilled water, followed by 2 minutes in 100% ethanol and 2 minutes in 70% ethanol. Staining solutions were diluted to 1:2 eosin and 1:3 methylene blue, each applied for 5 minutes. These improved steps were retained for subsequent assays.

The fourth assay tested the impact of acid concentration. Slides were prepared as before, but the denaturation step was adjusted to 0.1 M HCl for 5 minutes instead of 0.08 M. Lysis, neutralization, and staining followed the improved method from the third assay.

In the fifth assay, multiple slides were used to test combinations of denaturation and lysis times. One slide underwent 15 minutes of lysis with 0.1 M HCl for 5 minutes; another used 10 minutes of lysis followed by a longer acid exposure of 20 minutes. A third slide reversed the timing 15 minutes of lysis after 15 minutes of acid treatment. A fourth variation switched between lysis and acid treatment and extended the lysis time further for 20 minutes and kept the acid for 5 minutes as for the 5th slide, we switched between the lysis and acid

steps but we extended in both timing 20 minutes for HCL and 15 minutes for lysis treatment. All slides followed the improved neutralization and staining steps.

The sixth assay introduced a sequential acid exposure method. Two slides were prepared with alternating steps of acid and lysis. The first slide underwent 5 minutes of 0.1 M HCl, then 15 minutes of lysis, followed by a second 15-minute acid step. The second slide reversed this order with 15 minutes of acid, then 15 minutes of lysis, and a final 5-minute acid step. Both were then processed using the modified neutralization and staining protocol from the third assay.

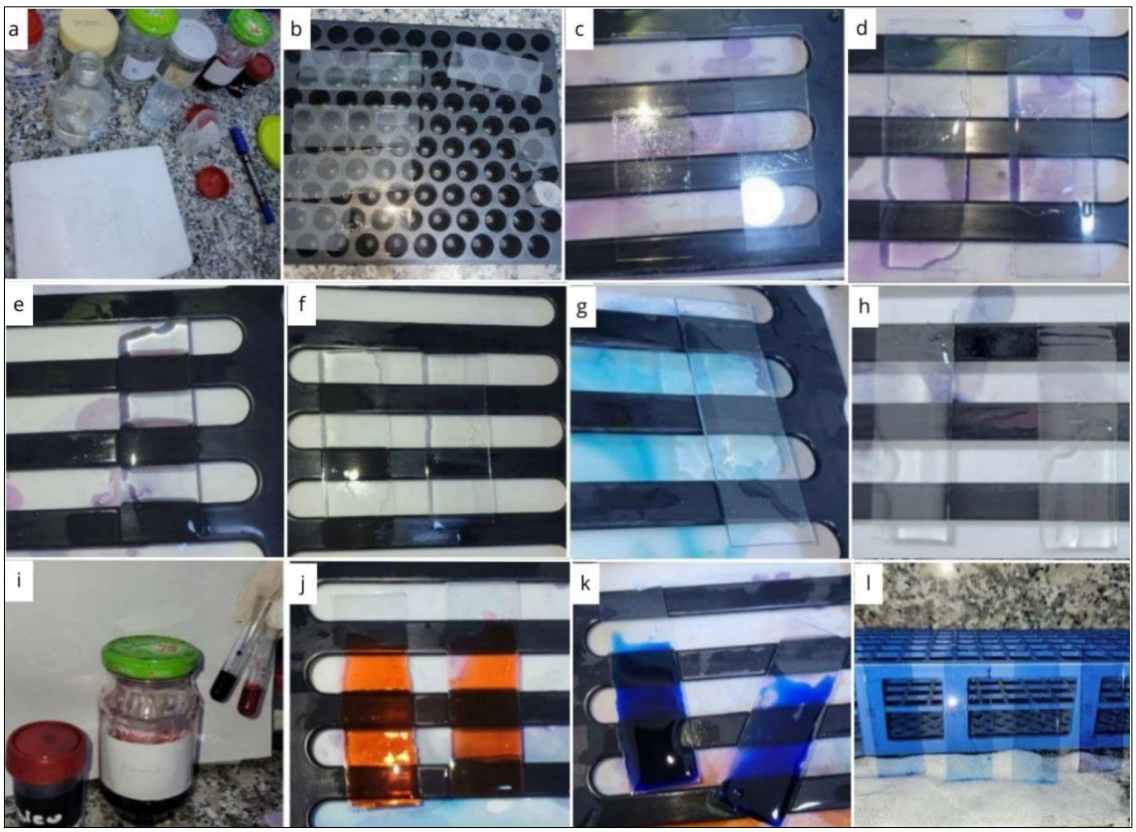

Figure 8: The Main Steps used in the Protocol

Preparation of materials and reagents (a) ; Slides embedded with agarose and sperm, placed in a solidifying tray for cooling at 4°C before removing coverslips (b) ; Close-up of solidified slides after coverslip removal (c) ; Application of acid denaturation step using HCl (d) ; Application of lysis buffer (e) ; Neutralization process using distilled water (f) ; 100% ethanol fixation step (g) ; 70% ethanol fixation step (h) ; Staining dilution (i) ; Staining with eosin (1/2 dilution) (j) ; Staining with methylene blue (1/3 dilution) (k) ; Stained slides are left to dry before microscopic examination (i)

(Original Picture)

Chapter III

Results and Discussion

III.1. Clinical Interpretation of Sperm DNA Fragmentation Test Findings

III.1.1. Overview of Study Population and Purpose

The 11 patients ages ranged from 29 to 43 years, with a mean age of 35.4 ± 4.8 years. All individuals' lifestyle, anthropometric characteristics, and marital status are detailed in Table 5 (In Appendices). These parameters include age, height, weight, smoking status, diet quality, and relationship status, temperature and cold exposure, standing position and were considered as potential influencing factors on DNA fragmentation levels.

III.1.2. Interpretation of Microscopic pictures and DFI Outcomes

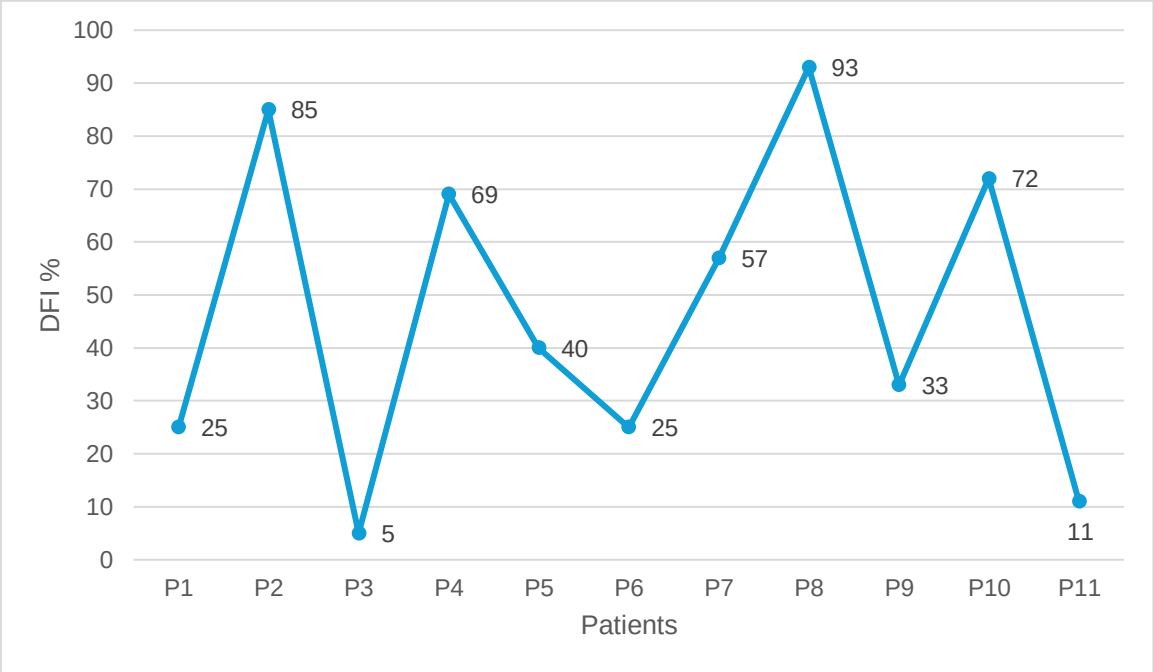

Figure 9: Interpretation of Sperm DNA Fragmentation Test using Halo Sperm Kit.

Figure (9) illustrate a graphical representation of DNA Fragmentation Index (DFI) levels of the patients, while Figure (10) shows microscopic images for each of the 11 patients. The results show a wide spectrum of sperm DNA integrity ranging from excellent to seriously compromised with fertility potential and clinical decision-making implications.

Severely deranged chromatin and high levels of DFI (≥30%) were observed in 7 out of 11 patients (P2, P4, P5, P7, P8, P9, P10).

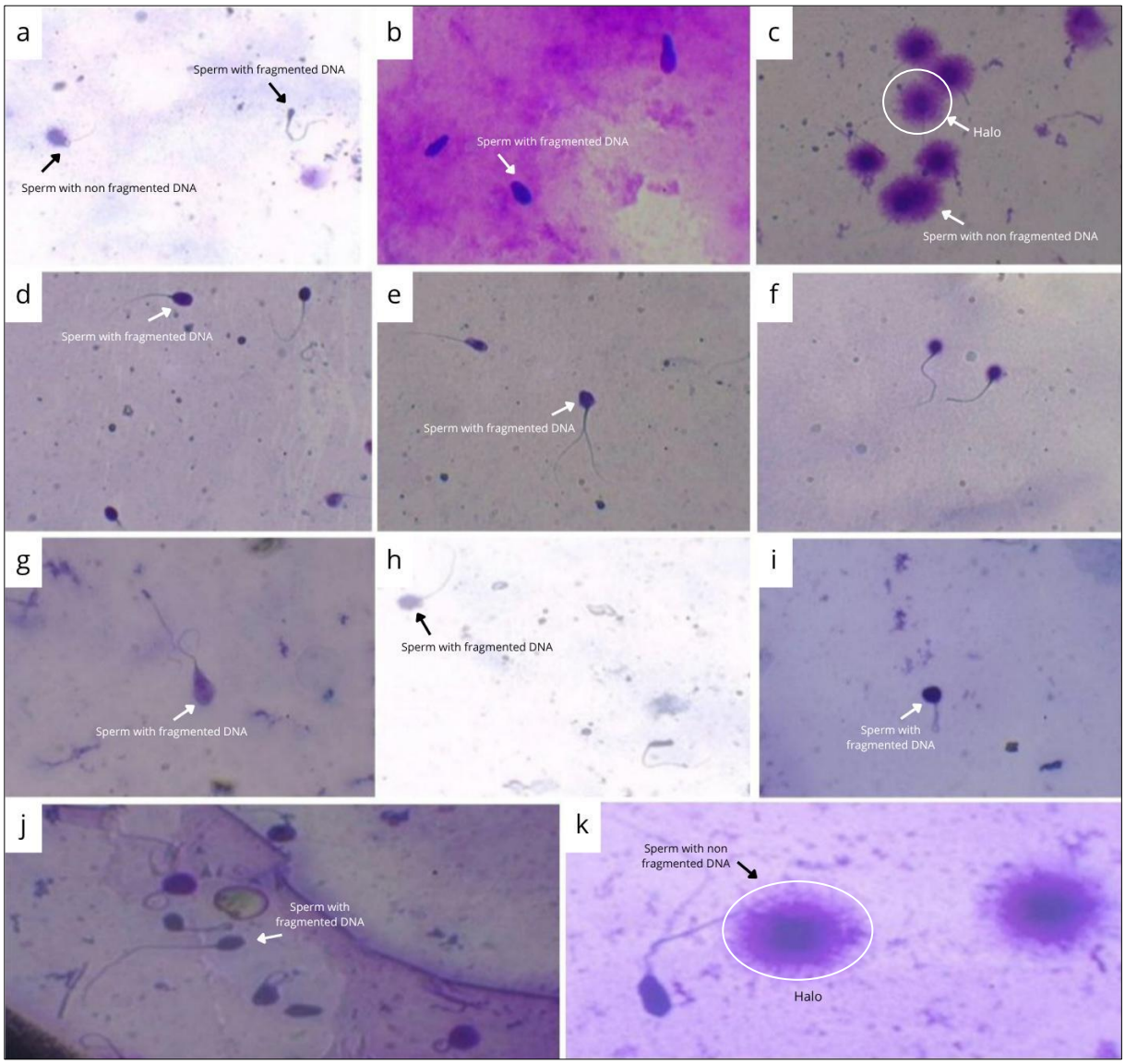

Figure 10: Microscopic Images of Sperm DNA Fragmentation in the Studied Population.

P1 (a) ; P2 (b) ; P3 (c) ; P4 (d) ; P5 (e) ; P6 (f) ; P7 (g) ; P8 (h) ; P9 (i) ; P10 (j) ; P11 (k)

(Original Picture)

Patients P3 (5%) and P11 (11%) exhibited low DNA fragmentation, indicating good sperm DNA quality, as supported by the presence of halos in microscopic images (Figure 9: c and k). These findings confirm their favorable prognosis for ART success, while also suggesting that these patients are apt to have causes of subfertility that are independent of DNA fragmentation emphasizing that the use of DFI should supplement, not supplant, routine semen analysis and clinical evaluation.

Moderate DFI (25%) was observed in patients P1 and P6, where mixed halos both fragmented and non-fragmented spermatozoa were present on microscopy (Figure 9: a and f), the inference was in favor of moderate quality DNA a borderline state which can perhaps still be improved upon by antioxidant treatment or change of diet.

P9 (33%) and P5 (40%) showed elevated fragmentation levels, with mixed sperm populations exhibiting both the presence and absence of halos (Figure 9: i and e), suggesting a decline in DNA quality.

Predominant lack of halos (Figure 9: g, d and b) was typically seen in patients with higher DFI P7 (57%), P4 (69%) and P2 (85%), consistent with the clinical explanation of grossly deranged chromatin. These samples incriminate compromise of fertilization potential and increased risk of miscarriage.

P10, with a past history of bilateral hernia and soon to be re-evaluated surgically, has a DFI of 72%. The simultaneous micrograph proves to bear evidence of widespread fragmentation (Figure 9 j), suggesting probable chronic ischemia or inflammation of testicular tissue.

P8, whose sperm microscopically exhibited no halo formation near total fragmentation (Figure 9 h), had a DFI of 93%, highlighting the extremity of the chromatin damage and perhaps relating to oxidative stress or other systemic issues.

Microscopic visual examination, when used in conjunction with DFI percentages, provides a forceful tool for patient education and therapeutic decision making.

III.1.3. Association Between Lifestyle Factors and DNA Fragmentation

III.1.3.1. Smoking

Figure (11) illustrates the proportion of patients who reported smoking habits, among the 11 male participants, 27% were smokers, while 73% were non-smokers.

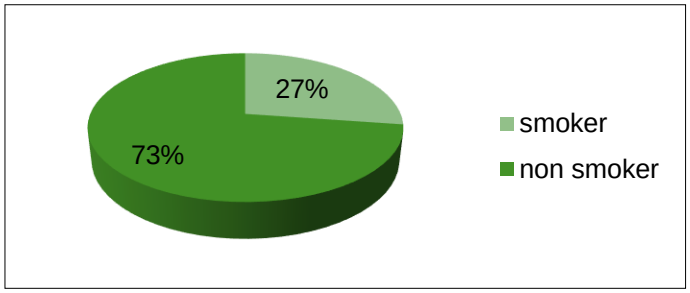

Figure 11: Distribution of Smoking Status Among the Studied Population.

Population based recent studies confirm that smoking significantly increases sperm DNA fragmentation, as well as decreased semen volume, motility, and morphology. Importantly, the values of DFI and zinc are elevated in heavy smokers with a marker of oxidative stress in seminal plasma (Omolaoye *et al*., 2022; Osadchuk, Kleshchev and Osadchuk, 2023).

In our study, P2 and P9, both smokers, exhibited high DFI values of 85% and 33%, respectively, aligning with the literature that links tobacco exposure to increased chromatin damage. Interestingly, P3, also a smoker, presented a low DFI value of 5%, suggesting that while smoking is a strong oxidative stressor, other factors may modulate its impact on DNA integrity.

These results directly support our findings high DFI patients also described smoking and unsound diets, underlining smoking as the greatest cause of chromatin damage through oxidative mechanisms (Omolaoye *et al.*, 2022; Osadchuk, Kleshchev and Osadchuk, 2023).

III.1.3.2. Diet

According to (figure 12) patients with unhealthy alimentation diet proportion constitute 46% (P1,2,5,8,9) their DFI level (25%,85%,40%,93%,33%), while it is 36% (P3,6,10,11) their DFI level (5%,25%,72%,11%) for patients with healthy alimentation diet, while 18 % of patients didn't provide information out of 11 patients.

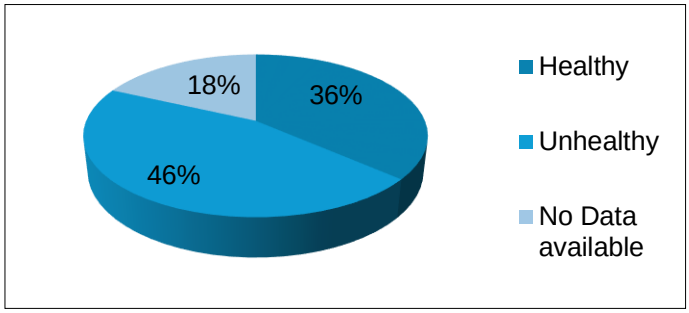

Figure 12: Distribution of Diet Quality Among Studied Population.

Recent research points to the fact that diet quality influences sperm DNA fragmentation directly. An RTC of low-carb, organic Mediterranean diet (rich in fresh vegetables, red fruits, whole foods, and healthy fats) reduced DFI from 44.2% to 23.2% over three months ($p < 0.005$) (Corsetti *et al.*, 2023). Systematic review established that Mediterranean dietary patterns rich in fruits, vegetables, whole grains, and healthy fats uniformly associated with improved seminal parameters, such as reduced DFI (Piera-Jordan *et al.*, 2024). On a molecular scale, unwholesome "Western" diets made of high levels of processed foods and saturated fats associate with greater oxidative damage and even altered sperm DNA methylation. (Tomada *et al.*, 2023) These outcomes strongly correlate with our finding: poor dietary patients exhibited elevated DFI, while healthier consumers showed lower DFI.

III.1.4.Clinical Conditions and Their Effect on Sperm DNA Integrity

In figure (13), In our study, varicocele was observed in 8 out of 11 patients. Specifically, 27% had no varicocele (P5, P7, P11), 46% had stage I varicocele (P1, P2, P4, P8 and P10), and 27% had advanced stage III varicocele (P3, P6 and P9). Notably, the presence and severity of varicocele appeared to correlate with elevated DNA fragmentation indices (DFI). For instance, P2 and P4, both with stage I varicocele, exhibited high DFI values (85% and 69%), while P6 and P9 despite having stage III varicocele, they had moderate DFI (25%, 30%) respectfully. Conversely, P3, who also had stage III varicocele, showed a very low DFI (5%), suggesting that individual variation and other modulating factors play important roles in sperm DNA stability.

Patients without varicocele presented diverse DFI outcomes. P11, with no varicocele and a DFI of 11%, showed excellent chromatin integrity. P5, also without varicocele, had a DFI of 40%, P7 with DFI of 57% potentially implicating other factors.

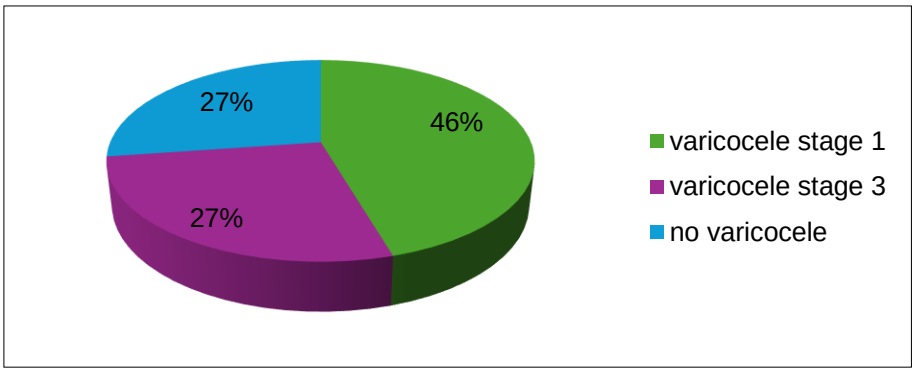

Figure 13: Prevalance and Staging of Varicocele Among the Studied Population.

Another 1,070 men with varicocele revealed a marked mean decrease in DFI (~ 7.2%) following varicocelectomy (Lira Neto *et al*., 2024). Consistent with our findings, the patients with varicocele also had elevated DFI suggesting that repair can decrease DNA fragmentation. Mechanistically, varicocele has been implicated to be associated with increased oxidative stress, underpinning this correlation (Abdulmassih Wood *et al*., 2021b).

Environmental and positional stressors further influenced fragmentation patterns. As illustrated in figure (14) patients P2 and P10, exposed to both cold temperatures and prolonged standing, had very high DFI levels (85% and 72%). P3, exposed to cold, heat, and standing, showed minimal DFI (5%). P4, P5, and P6, all exposed solely to prolonged standing, exhibited moderate to high DFI (69%, 40%, and 25%). P9, with both temperature exposure and standing, had a DFI of 33%.

These findings suggest that varicocele especially when combined with thermal or postural stress may amplify oxidative damage and compromise chromatin integrity.

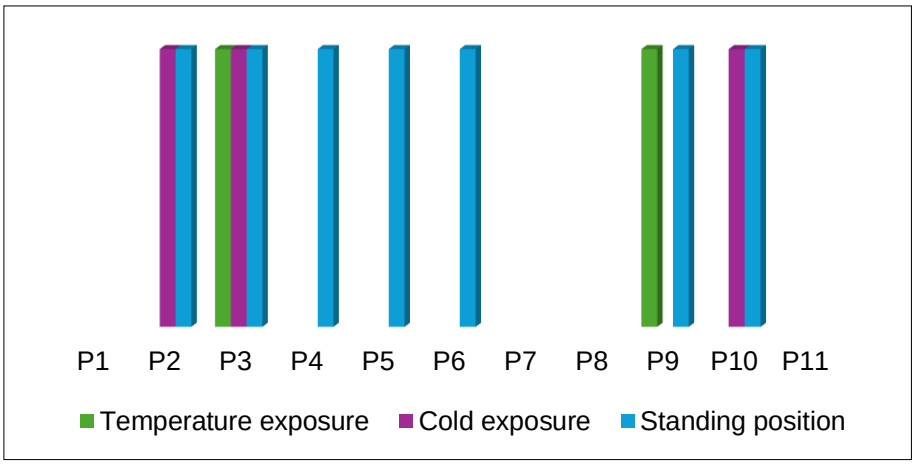

Figure 14: Environmental and Postural Exposures Among Studied Population.

Very similar to this, scrotal heat stress from prolonged standing, tight pants, or hot work environments has been linked to elevated DFI, even minimal 2–3°C increases in scrotal temperature can cause oxidative stress to sperm (Zhang *et al*., 2015). Additionally, hydrocele a condition which increases scrotal temperature due to fluid accumulation can impair testicular thermoregulation by trapping heat and may cause DNA fragmentation (O'Reilly *et al*., 2016). Studies also confirm that prolonged sitting time or occupational standing increases testicular temperature, mimicking varicocele dysfunction (McKinnon *et al*., 2022). In our findings, the varicocele patients or those who also experienced prolonged standing and heat exposure showed significantly high DFI, corroborating these findings.

III.1.5. Reproductive Outcomes and Predictive Value of DFI

According to (figure 15) there are 9 married patients so 82% that consulted for infertility, 2 of them P7 and P8 had encountered miscarriage problems presenting a DFI level of 57% and 93%, 2 single male that consulted for different issues so 18% out of 11 patients.

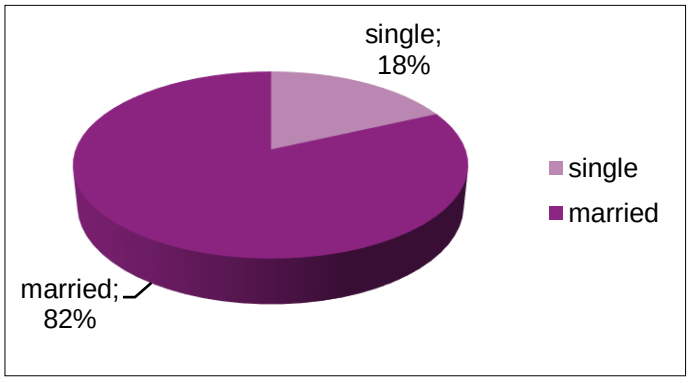

Figure 15: Marital Status of Study Participants

Studies by Stavros *et al*., (2024) and Ziouziou *et al*., (2024). concluded that elevated sperm DNA fragmentation lowers natural conception rates, risk of miscarriage, and diminishes ART success especially in patients with unexplained infertility or recurrent pregnancy loss. Our cohort men with high DFI consistently reported infertility or miscarriage, confirming the diagnostic value of DFI testing for reproductive counselling and planning.

III.1.6. Pathological Profiles and Sperm DNA Damage

Severe oligo-astheno-teratozoospermia (OAT) with necrospermia indicates very low sperm count, poor motility, abnormal morphology, and excessive dead sperm (Brahem *et al*., 2012). A research study from Iran comparing controls and OAT patients indicated that OAT is strongly related to profoundly elevated sperm DNA fragmentation (as measured by both TUNEL and SCD assays) and increased lipid peroxidation markers of oxidative stress (Chegini *et al*., 2024). Clinically, they are associated with our cohort's high DFI values and suggest underlying oxidative damage and DNA fragmentation.

Inguinal hernias, particularly those that affect the spermatic cord or disrupting scrotal blood flow, can negatively affect sperm production and testicular perfusion (Lolah *et al*., 2018). A randomized clinical trial determined that testicular blood flow and sperm parameters may deteriorate after surgery but become normal upon repair (Damous *et al*., 2023). For our candidate patient (P10), chronic or recurrent hernia may contribute to compromised peri-testicular function, possibly worsening DFI.

III.2. Optimization and Evaluation of a Developed Protocol

III.2.1.Objectives and Design of Protocol Development

To complement the clinical investigation, a secondary objective of this study was to develop and optimize local sperm chromatin dispersion (SCD) protocol using four additional semen samples. These samples were not part of the 11-patient diagnostic cohort and were used exclusively to replicate, test, and improve upon the commercially available Halo Sperm kit. The development aimed to create a more accessible, cost-effective alternative suitable for routine laboratory use, particularly in resource-limited settings. Each trial was designed to isolate specific variables within the SCD procedure, such as the agarose medium, lysis solution, acid denaturation, fixation, and staining conditions.

III.2.2.Summary of Assay Progression and Adjustments

III.2.2.1. Assays 1 and 2: Testing Agarose Medium and Slide Clarity

III.2.2.1.1. First assay

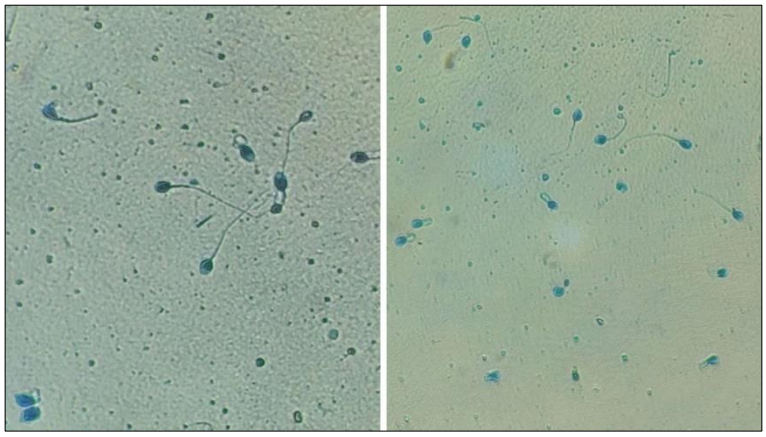

Figure 16: Microscopic image of agarose slides from the primary protocol

(Original Picture)

Sperm cells on agarose slides showing a clear outline of the sperm cells are visible, with no halo formation observed.

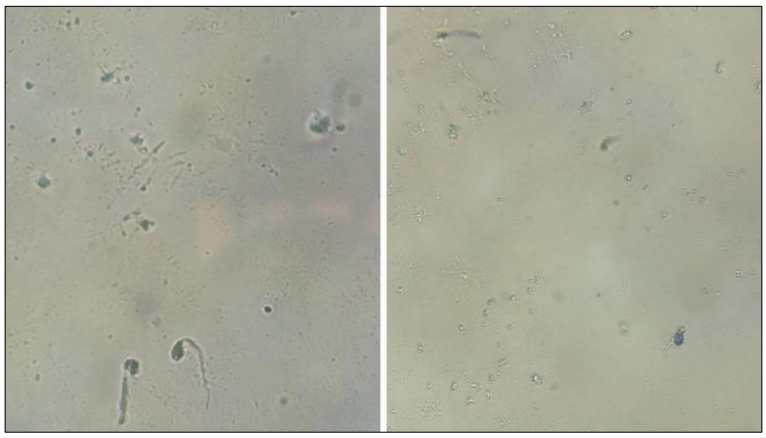

Figure 17: Microscopic image of nutrient agar slides from the primary protocol

(Original Picture)

When replacing agarose with nutrient agar poor results were obtained.

The target in the first assay was to test the primary protocol elaborated after many research done to see if it succeeds to show the halos, and prove the effectiveness of the prepared agarose and that by comparing the results of using the nutrient agar to the results of agarose as it shown that using agarose was way more effective in showing the outlines and fixating the spermatozoa, though going with the next steps to the final result of the assay it failed to show any halos in both cases.

III.2.2.1.2. Second assay

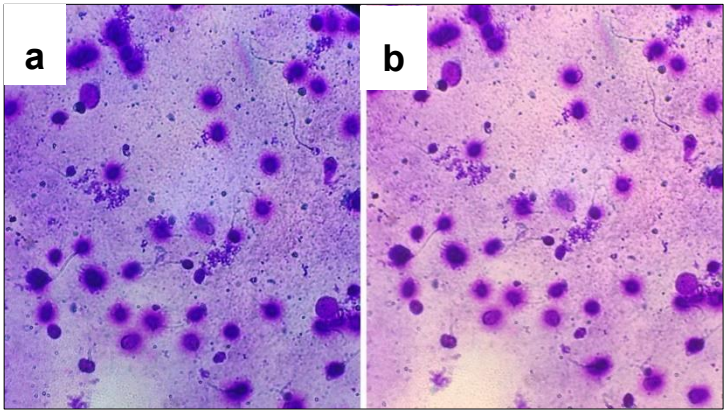

Figure 18: Microscopic image of the 1st slide from the 2nd assay

Prepared agarose and Halosperm kit (a) ; Full kit Halosperm steps (b)

(Original Picture)

When replacing the HaloSperm kit's agarose with our prepared agarose while keeping the rest of the protocol unchanged, clear results were obtained. Spermatozoa were visible, with some exhibiting halo formation. Similar observations were made using the complete commercial kit; however, the results with our agarose showed better clarity and contrast.

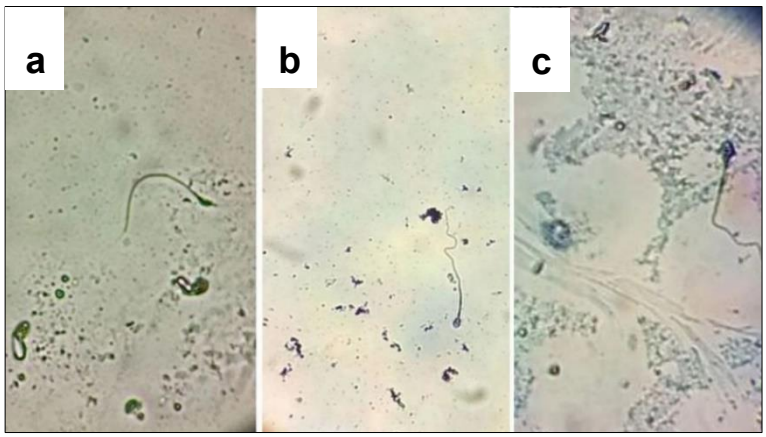

Figure 19: Microscopic image of the 2nd, 3rd, 4th slide from the 2nd assay

2nd slide (a) ; 3rd slide (b) ; 4th slide (c)

(Original Picture)

Clear results were observed in the second slide, where only our prepared agarose and the semen sample were used, indicating that the agarose effectively fixed the sample. In the third slide, where our complete protocol was applied using our prepared agarose and all the remaining steps of the Halo Sperm kit, a clear outline of the sperm cells was obtained with no halo formation observed. In the fourth slide, where a diluted semen sample was processed using the same protocol, sperm cells also showed a clear outline with no visible halo.

For the second assay just as the first the target was also to test the primary protocol and prove the effectiveness of the agarose product by comparing the results to the agarose of

Halo Sperm Kit in the first slide and it was successfully proved very effective as well as clear halos, as for the other slides where we followed the primary protocol it again failed to show any halos leading us to think there is a problem in the next steps so we made modifications and adjusted the protocol on the next assays to achieve a final successful result.

III.2.2.2. Assay 3: Buffer Validity and Denaturation Variables

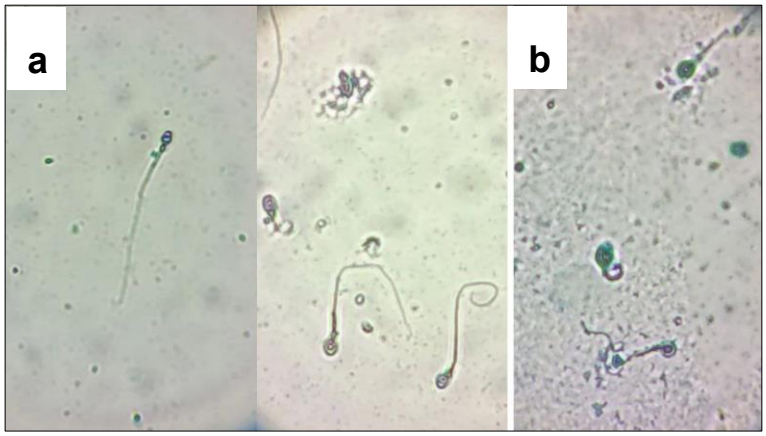

Figure 20: Microscopic image of the 1st, 2nd and 3rd slides from the third assay

1st slide (a) ; 2nd and 3rd slide (b)

(Original Picture)

In the first slide, the lysis buffer was replaced with Reagent 1 from the Halo Sperm kit, while the neutralization, fixation, and staining steps were substituted with our own protocol. A clear outline of the sperm cells was obtained, with no halo formation observed. In the second and third slides, our full protocol was applied. In the second slide, clear outlines of the sperm cells were observed without halo formation; however, crystallization occurred due to the slide being left to dry after the lysis buffer was removed. In the third slide, a clear outline of the sperm cells was again obtained with no visible halo.

In the third assay, we aimed to test whether the lysis solution was responsible for the lack of halo formation observed in our sperm DNA fragmentation tests. To confirm this, we ran a comparative slide using our protocol alongside another where we replaced our lysis buffer with 1st reagent from the commercial Halo sperm kit. Both slides produced similar results, with no halos detected, suggesting that the problem did not originate from the lysis buffer.

Although our exact buffer composition (1% SDS, 0.5 M NaCl, 10 mM Tris HCl) is somewhat unique, it fits within the range of validated SCD protocols. For instance, Absalan *et al*., (2011) reported effective use of (0.4 M Tris, 0.8 M DTT, 1% SDS, 2 M NaCl, 0.05 M Triplex) in producing reliable sperm chromatin dispersion. These examples confirm that our buffer's core components are functionally sound, reinforcing that the observed lack of halos likely stemmed from procedural variables rather than chemical insufficiency. However, when compared with the protocol developed by Fernández *et al*., (2003) several differences are evident. Their method utilizes a two-step lysis and neutralization process: first with a solution containing (0.4 M Tris, 0.8 M DTT, 1% SDS, and 50 mM EDTA) for 10 minutes, followed by a second solution containing (0.4 M Tris, 2 M NaCl, and 1% SDS) for 5 minutes By contrast, our buffer lacks both DTT and EDTA, which may partially account for its lower efficiency in promoting halo formation particularly under suboptimal acid denaturation or timing conditions. However, the comparable outcome observed with the commercial buffer suggests that the issue may not lie solely in the chemical composition.

III.2.2.3. Assay 4: Improved Fixation and Staining Adjustments

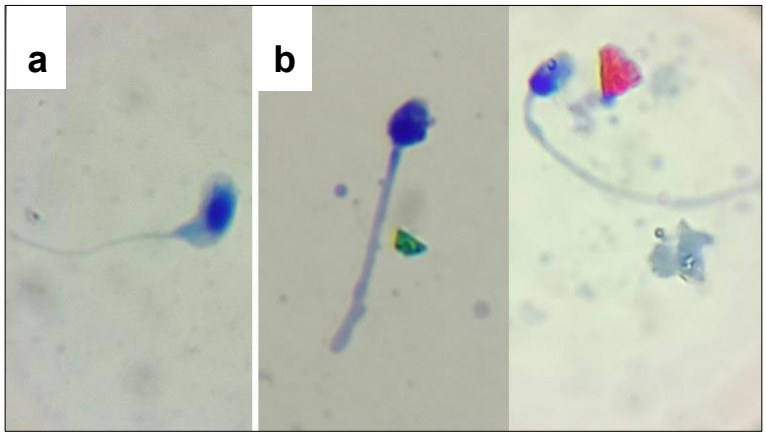

Figure 21: Microscopic image of the 1st and 2nd slides from the fourth assay

1st slide (a); 2nd slide (b)

(Original Picture)

In both slides, the same modifications to the neutralization, fixation, and staining steps were maintained, with an additional adjustment of increasing the HCl concentration from 0.08 M to 0.1 M. The results demonstrated good drying and proper agarose setting, along with effective fixation. Clear outlines of the sperm cells were observed in both cases, with no halo formation detected.

The results of the 3rd assay pointed us toward procedural variables as critical factors, prompting us in the fourth assay to investigate the impact of acid treatment conditions more closely. To address potential procedural issues, adjustments were made to the fixation and staining steps. The neutralization and fixation protocol were modified to follow the Halo sperm procedure, involving sequential immersion in distilled water, 70% ethanol, and 100% ethanol. This change led to an observable improvement in fixation quality, which is essential for preserving the structural integrity of sperm nuclei during analysis. Furthermore, the initial staining concentrations were reconsidered; eosin and methylene blue were diluted to 1:2 and 1:3, respectively, to correct the excessive intensity that had previously hindered chromatin visualization. These refinements enhanced the clarity of the stained slides and reduced background noise, although they did not result in halo formation.

Therefore, the lack of halo in our assay likely stems from other parameters, which led us to shift our focus in the fourth assay toward optimizing the acid denaturation step by increasing the HCl concentration from 0.08 M to 0.1 M, while keeping the improved neutralization, fixation, and staining conditions. This adjustment produced a subtle yet notable change in chromatin dispersion: rather than clear halos, we observed a darker central core surrounded by faint light-blue staining. While this pattern did not match the classical halo morphology, it did indicate partial DNA loop relaxation suggesting that acid concentration was influential, but exposure time was likely insufficient for complete DNA release. Moreover, Fernández *et al*., (2003) emphasized that while acid denaturation is essential for protein removal and DNA decondensation, optimal halo formation requires both adequate acid strength and precise timing. Given that our fixed buffer conditions had proven effective, this emerging pattern highlighted the need to systematically optimize the denaturation time.

III.2.2.4. Assay 5: Optimal Timing of Denaturation and Lysis

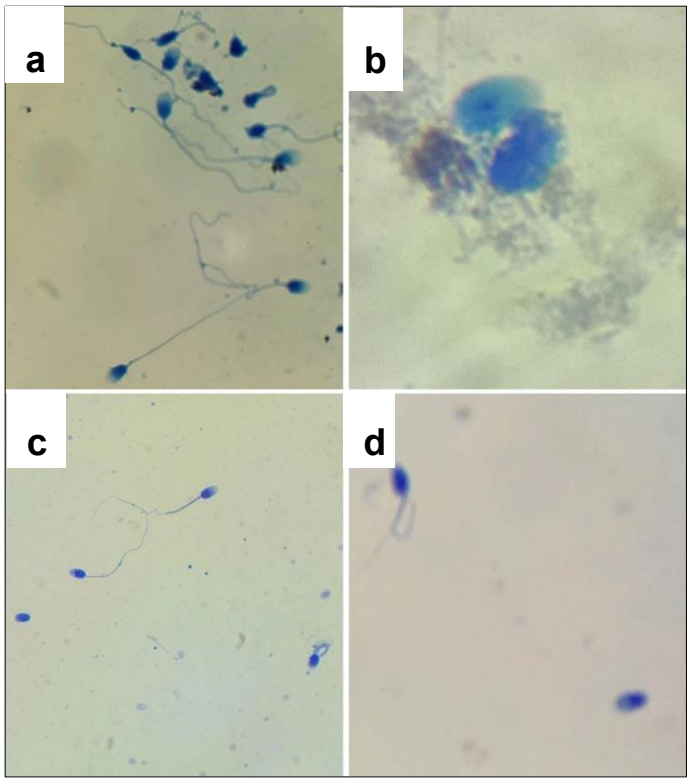

Figure 22: Microscopic image of the 1st, 2nd, 3rd and 4th slides of the fifth assay

1st slide (a); 2nd slide (b); 3rd slide (c); 4th slide (d)

(Original Picture)

In this assay, the modifications from the fourth trial were maintained, with a change in timing applied. In the first three slides, the lysis buffer treatment preceded the HCl application, whereas in the fourth slide, the order was reversed, with HCl applied prior to the lysis buffer. In the first slide, clear outlines of the spermatozoa were observed. Some cells exhibited intensely stained heads with no detectable halo, while others showed a gradient of staining intensity darker near the neck region adjoining the flagellum and lighter, bluish staining in the apical region of the head. The second slide presented poorly defined contours with overall light blue staining. Although a halo-like zone appeared, it could not be confirmed as true halo formation due to the absence of a clearly defined sperm head within the structure. In both the third and fourth slides, clear outlines of the spermatozoa were observed. As in the first slide, some cells showed intensely stained heads without detectable halos, while others exhibited a gradation in staining intensity from the neck to the head.

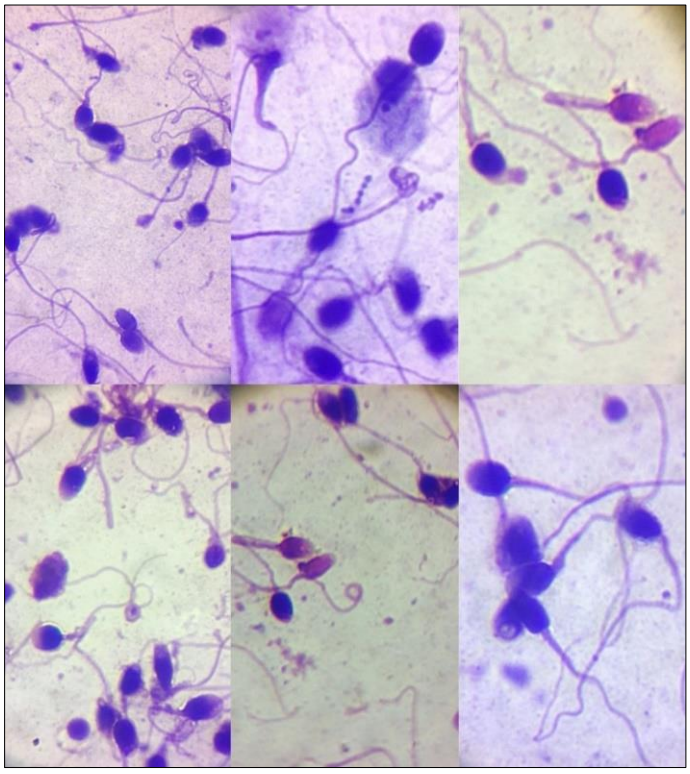

Figure 23: Microscopic image of the 5th slide of the fifth assay

(Original Picture)

In the fifth slide, the same protocol as in the fourth was followed, with the addition of an extended HCl incubation time. Well-defined outlines of the spermatozoa were observed. Some cells exhibited clear halo formation, while others did not; nonetheless, the overall results were distinctly visible and well preserved.

In this assay after many trials to change and adjust the products and steps procedures in the previous assays, it was decided that the next adjustment to test is to add time for them in the first part; we noticed some slides began to show a sign getting affected.

For the second part after switching between the cell lysis and acid treatment steps as well as adding time to both we finally came to a good result in the fifth slide where clear halos were noticeable leading us to conclude that time and the flow of steps is crucial in this test.

III.2.2.5. Assay 6: Sequential Acid Treatments

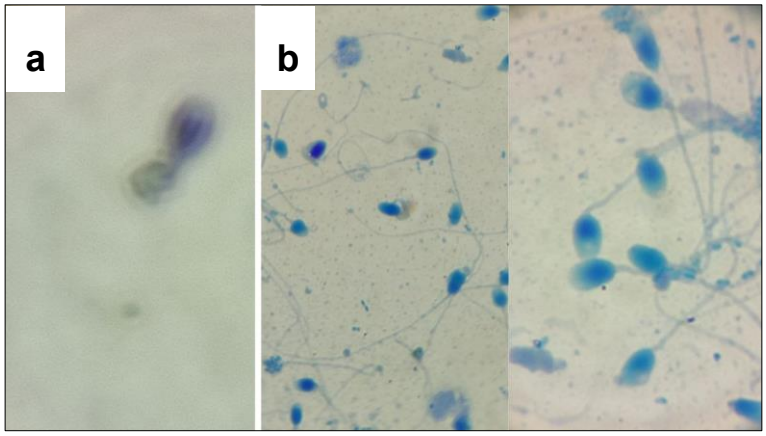

Figure 24: Microscopic image of the 1st and 2nd slides of the sixth assay

(Original Picture)

1st slide (a); 2nd slide (b)

In both slides, a sequential method was tested, consisting of HCl treatment followed by lysis buffer, then a second HCl application, with variations in incubation times. In the first slide, poor results were obtained. In contrast, the second slide showed clear outlines of the spermatozoa. Some cells exhibited intensely stained heads with no detectable halo, while others displayed a gradation in staining intensity darker near the neck region adjoining the flagellum and lighter, bluish staining in the apical region of the head.

In the sixth assay, we explored whether a sequential acid treatment before and after the lysis step could enhance chromatin dispersion. This approach was motivated by the outcome of the fifth assay, particularly slide 5, which showed that a prolonged acid exposure yielded the most promising halo formation. Two sequential protocols were tested neither protocol produced visible halos. These findings reinforce that continuous, uninterrupted acid denaturation is essential for achieving proper chromatin dispersion. Splitting the acid exposure into two phases did not enhance halo formation and may, in fact, hinder DNA loop release. Another study of Fernández *et al.*, emphasizes that the acid solution must be carefully timed to allow selective DNA denaturation and optimal halo distinction (Fernández, Johnston and Gosálvez, 2018). Accordingly, our sixth assay confirmed that both sequential acid treatments failed to match the halo clarity seen with the continuous 20 min HCl treatment from the fifth assay. This underscores that time-based procedural fidelity during acid

denaturation remains the critical determinant for achieving full DNA loop release and clear halo formation in SCD protocols.

III.2.3. Final Protocol Evaluation and Limitations

Consequently, our results support the conclusion that the optimal protocol remains the continuous 20 min HCl exposure followed by 15 min lysis solution treatment, as implemented in the fifth assay. This protocol yielded the most clearly defined halos and thus represents the most reliable configuration for our experimental setup.

Table II: Finalized Protocol Developed for Sperm DNA Fragmentation Analysis

	Sample Embedding	Acid Treatment	Cell Lysis	Neutralization and Fixation	Staining
Final Protocol	Mix 50 µL of sperm sample with 100 µL of agarose (1% at 37°C). Place a drop on a clean slide and gently place the coverslip on the drop. Allow to solidify at 4°C for 5 minutes. Remove the coverslip. Let it dry for 15min.	Immerse the slide in 0.1M HCl for 20 minutes at room temperature.	Immerse the slide in lysis buffer (1% SDS, 0.5M NaCl, 10mM Tris-HCl) for 15 minutes at room temperature.	Immerse the slide in distilled water for 5 minutes. Immerse in 70% ethanol for 2 minutes. Immerse in 100% ethanol for 2 minutes.	Immerse in eosin Y for 5 minutes, then rinse with distilled water. Immerse in methylene blue for 5 minutes, then rinse and allow to dry.

Conclusion, Recommendations and Perspectives

CONCLUSION, RECOMMENDATIONS and prospectives

This study aimed to develop a reliable, manual protocol for detecting sperm DNA fragmentation using the SCD assay and evaluate its clinical relevance. Through adjustments to denaturation, lysis, incubation, and staining steps, we achieved consistent and clear chromatin halo visualization.

However, a key limitation of this study was the limited number of semen samples (4 samples) available for protocol development. This restricted the ability to test the method across a wider range of sample conditions and variations. Additionally, while we aimed to explore potential causes of DNA fragmentation by incorporating further diagnostic or biochemical steps into the protocol, this was not possible due to the unavailability of certain essential reagents.

Nevertheless, the protocol developed shows great promise as a cost-effective and adaptable alternative to commercial kits like Halo Sperm. Although it requires slightly more time, it remains practical for use in local laboratories, especially where access to commercial kits is limited.

Based on these findings, we recommend testing the protocol on a larger sample size to ensure its robustness and reproducibility. Incorporating sperm DNA fragmentation testing as a complementary diagnostic tool particularly in cases of unexplained infertility, repeated ART failure, or miscarriage could provide deeper insight into male fertility issues.

Future experiments should include additional biochemical steps to help elucidate the underlying mechanisms of DNA fragmentation, including oxidative stress markers or chromatin packaging defects.

It would be interesting, to validate the protocol on a broader population and correlate DNA fragmentation levels with fertility outcomes, such as pregnancy rates and ART success. Compare the effectiveness and sensitivity of the optimized SCD protocol with other DNA fragmentation assays such as TUNEL or Comet. Finally, it is essential that future research efforts contribute to the establishment of standardized norms and reference values for sperm DNA fragmentation testing, in order to ensure its clinical reliability and integration into routine fertility assessments.

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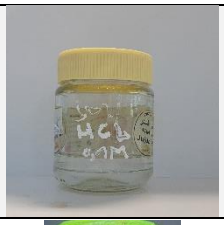
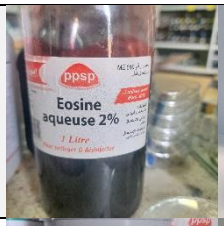
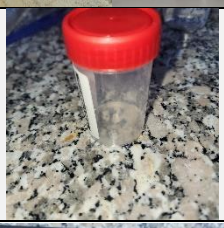
Appendices

APPENDICES

<u>Table III:</u> Reagents and Material

REAGENT AND MATERIALS	PICTURE
AGAROSE	
NUTRIENT AGAR	
LYSIS BUFFER	
HCL 0.08 M	
HCL 0.1 M	
PBS	
ETHANOL 100%	
ETHANOL 70%	

APPENDICES

EOSIN Y	 
METHYLENE BLUE	 
SPERM SAMPLE	
SLIDES AND COVERSLIPS	
MICROPIPETTE	
STERILE TUBES AND MICRO TUBES EPPENDORF	
PH TEST ROLL	

APPENDICES

PORTE-LAMES	
BIOSAFETY CABINET	
BALANCE	
PHOTONIC MICROSCOPE	

Figure 25: Halo Sperm Kit

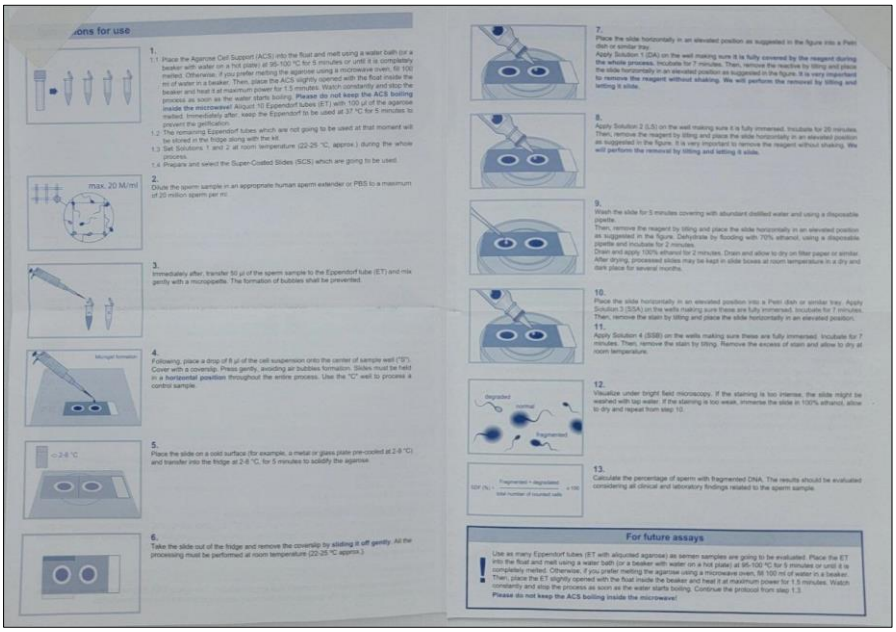


Figure 26: Halo Sperm Kit Protocol

APPENDICES

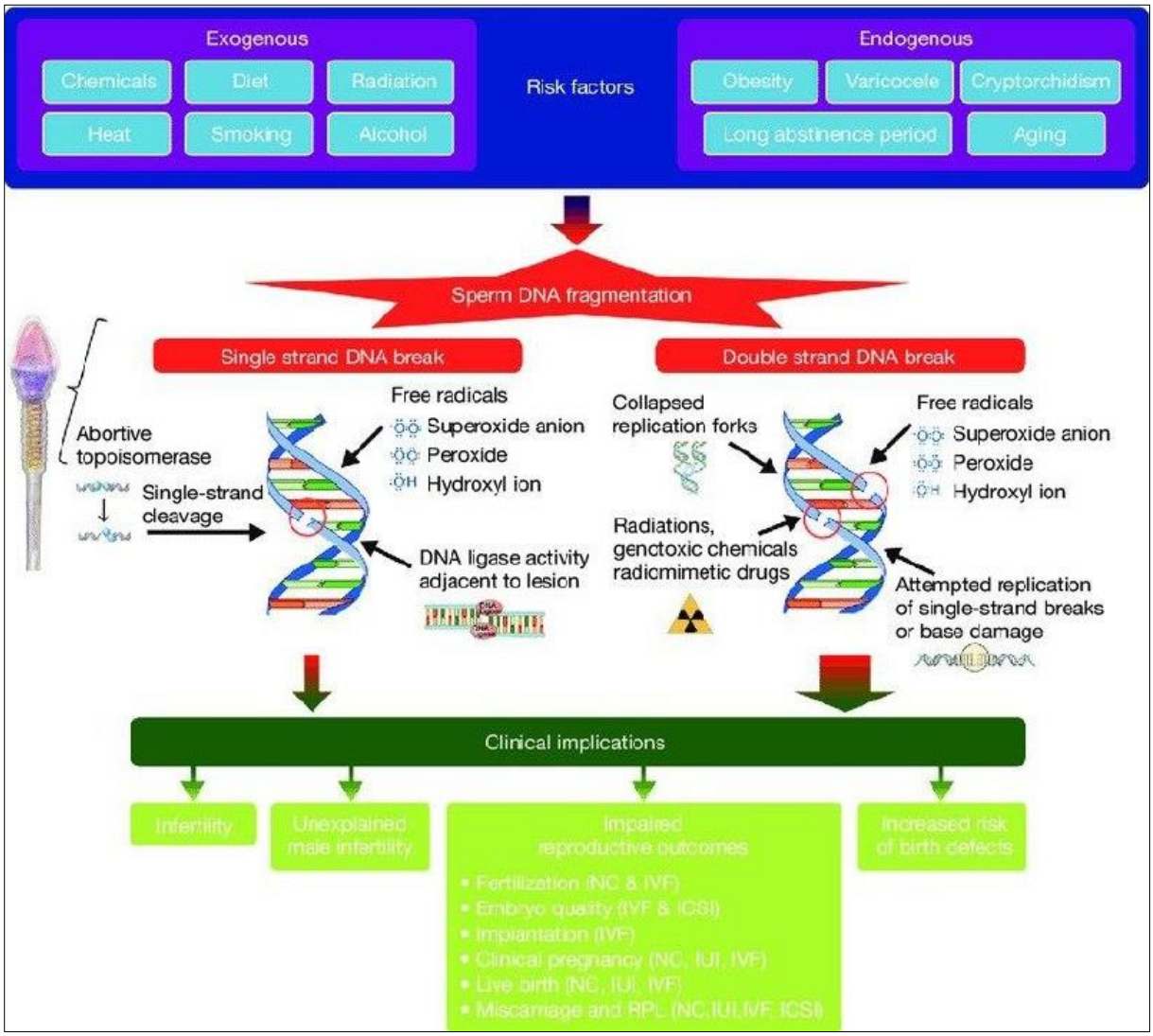


Figure 27: Summary of Risk Factors, Molecular Mechanisms of Sperm DNA Fragmentation and its Clinical Implications (Farkouh *et al*., 2022)

Parameter	**Lower limit (range)**
Sperm volume (ml)	1.5 (1.4-1.7)
Total sperm count (million/ejaculate)	39 (33-46)
Sperm concentration (million/ml)	15 (12-16)
Total sperm motility (PR NP)	40 (38-42)
Progresive sperm motility (PR,%)	32 (31-34)
Vitality (viable sperm,%)	58 (55-63)
Sperm morphology (normal forms,%)	4 (3.0-4.0)

Figure 28: The normal Values of Semen Analysis (Bartl *et al*., 2021)

APPENDICES

Table IV: Factors Influencing Sperm DNA Fragmentation Levels

	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11
Age	31	29	33	41	32	34	36	39	30	43	
Height		1.75 m	1.75 m		1.78 m	1.84 m	1.70 m	1.70 m	1.81 m	1.80 m	1.80 m
Weight		85 kg	70 kg		100 kg	94 kg	79 kg	68 kg	70 kg	79 kg	90 kg
Smoker	-	+	+	-	-	-	-	-	+	-	-
Alimentation Diet	Unhealthy	Unhealthy	Healthy		Unhealthy	Healthy		Unhealthy	Unhealthy	Healthy	Healthy
Single	+	+	-	-	-	-	-	-	-	-	-
Married	-	-	+	+	+	+	+	+	+	+	+
Temperature exposure		-	+	-					+	-	
Cold exposure		+	+	-					-	+	
Standing position		+	+	+	+	+			+	+	

Table V: Clinical Indications for Sperm DNA Fragmentation Testing

	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11
Varicocele	+	+	+ (Stage III)	+	-	+ (Stage III)	-	+	+ (Stage III)	+	-
Hydrocele	+	-	-	-	-		-	-	-	-	-
Infertility	-	-	+ (2Y)	+ (4Y)	+ (1½Y)	+ (1½Y)	+ (Stage II)	+	+ (3Y)	+ (3Y)	+ (1Y)
Miscarriage	-	-	-	-	-	-	• 2016–2018: Two miscarriages. • 2018: Birth of first daughter.	6 years of trying to conceive with 2 miscarriages.	-	-	-

APPENDICES

							• 2019: One miscarriage. • 2020: Birth of second daughter. • Post-2020: 2 miscarriages with complications, leading to the removal of one ovary.				
Spermogram							[5 × 10^6/ml]	[12 × 10^6/ml]	[20 × 10^6/ml]	[100 × 10^6/ml]	
Disease	Severe OAT and Necrospermia.	-	-	-	-	-	-	-	-	• Bilateral longitudinal hernia. • 2016: Underwent surgical repair. • Currently: Candidate for another surgical intervention.	-

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Theme:

Sperm DNA Fragmentation in Male Infertility: Clinical Evaluation by Halo Sperm test and Protocol Development

Presented by:

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