INFLUENCE OF VARIOUS FACTORS ON FUNCTIONAL AND MOLECULAR ASPECTS OF THE SEMEN QUALITY ASSOCIATED WITH MALE INFERTILITY

Thesis Submitted for the Award of the Degree of

DOCTOR OF PHILOSOPHY

in

Molecular Biology and Genetic Engineering

By

Kale Sudarshan Nagorao

Registration Number: 11720095

Supervised By

Dr Ashish Aggarwal (28916)

Associate Professor
Department of Biotechnology
School of Bioengineering and Biosciences
Lovely Professional University
Phagwara, Punjab (India)

Co-Supervised By

Dr. Jibanananda Mishra

Professor/Dean
School of Biosciences
RIMT University
Mandi Gobindgarh, Punjab (India)

LOVELY PROFESSIONAL UNIVERSITY, PUNJAB.

2025

DECLARATION

I, hereby declare that the work presented in this thesis, entitled "**Influence of various factors on functional and molecular aspects of the semen quality associated with male infertility",** submitted in partial fulfilment of the requirements for the degree of **Doctor of Philosophy (Ph. D.),** is the result of original research work carried out by me under the supervision of **Dr. Ashish Aggarwal,** Associate Professor, School of Bioengineering and Biosciences, Lovely Professional University, Punjab, India, and co-supervision of **Dr. Jibanananda Mishra,** Professor and Dean, School of Biosciences, RMIT university, Punjab, India.

In accordance with the general practice of reporting scientific observations, appropriate acknowledgments have been made wherever the work is based on the findings of other investigators. I further declare that this work has not been submitted, either in part or in full, to any other university or institute for the award of any degree.

(Signature of Scholar)

Name of the scholar: Kale Sudarshan Nagorao

Registration No.: 11720095

Department/school: Molecular Biology and Genetic Engineering

Lovely Professional University, Punjab, India.

CERTIFICATE

This is to certify that the work reported in the Ph. D. thesis entitled "**Influence of various factors on functional and molecular aspects of the semen quality associated with male infertility**", submitted in fulfillment of the requirements for the reward of degree of **Doctor of Philosophy (Ph.D.)** in **Molecular Biology and Genetic Engineering**, is the original research work carried out by **Kale Sudarshan Nagorao (11720095)** under our supervision,

We further certify that this is a bona fide record of his independent research and that no part of this thesis has been submitted for any other degree, diploma, or equivalent qualification.

(Signature of Supervisor)
Name of supervisor:
Dr Ashish Aggarwal
Associate Professor
School of Bioengineering and Biosciences
Lovely Professional University
Phagwara, Punjab (India)

(Signature of Co-Supervisor)
Name of Co-Supervisor:
Dr Jibanananda Mishra
Professor and Dean
School of Biosciences
RMIT university, Mandi
Govindgarh, Punjab (India)

Abstract

Infertility is a common concern that impacts millions of couples globally. Both male and female equally responsible for infertility. Male infertility rates steadily increasing year by year. Abnormal spermatogenesis and spermiogenesis are major causes of male infertility. Various factors responsible for impairing the normal semen parameters, including Health factor, lifestyle factors, diet factors, sex related behavioral factors, and environmental factors, contribute to male infertility. Health factors including age, body mass index (BMI), Lifestyle aspects such as addiction of alcohol/tobacco/smoking, stress, sleep quality, physical activity, and diet appears to significant ly impact semen quality in infertile men. Infertility appears to result in considerable social and psychological impacts, leading to anxiety, a sense of feeling helpless, and embarrassment. Although assisted reproductive technologies provide promise, their limits in accessibility and expense exacerbate mental stress for couples pursuing treatment. Assessment of male infertility involves evaluating reproductive history and conducting semen quality analysis. Semen analysis, a vital laboratory test, measures various physical and microscopic parameters to assess semen quality. Additional tests, such as the Sperm DNA fragmentation detection by chromatin dispersion (SCD) method, provide insights beyond standard parameters. These tests reveal information about DNA fragmentation, a crucial aspect of sperm quality. Considering the increasing incidence of male infertility and the considerable impact of health factor, lifestyle factors and environmental factors on sperm quality, it is necessary to investigate the complex relationships between these factors and male infertility. This study aims to evaluate the influence of multiple factors, such as age, BMI, lifestyle, diet and environmental exposer factors on the functional and molecular aspects of semen quality associated with male infertility. Basic semen analysis has been performed for assessment of macroscopic and microscopic semen parameters. For assessment of molecular aspects SCD test has been executed. The SPSS software used for data analysis.

The study collected semen samples from Indian infertile men at a fertility clinic, along with detailed information on their lifestyle and medical history. Data analysis using advanced statistical approaches will reveal substantial relationships between

components and semen quality measures. The discoveries of this study partake therapeutic implications as they highlight the importance of a comprehensive approach to diagnose male infertility, considering both functional and molecular aspects of sperm quality. This method appears to assist in identifying people that may gain from particular measures., such as antioxidant therapy or sperm selection procedures, and provide valuable information for couples seeking fertility counseling. The study also improves understanding of male infertility's etiology and pathophysiology, which appears to inform preventive and treatment techniques. The research encompasses the assessment of various factors, including age, BMI, and lifestyle, on the functional and molecular aspects of semen quality associated with male infertility. The study utilizes data from the Femcare Fertility center in Pune, Maharashtra, India. Prior permission was obtained, and a questionnaire was administered to collect relevant information before sample collection. Semen samples were collected, stored, and maintained following WHO guidelines to ensure sample quality and integrity. Well-equipped ivf labs were necessary for complete semen analysis. Physical and microscopic parameters were analyzed, including sperm count, motility, morphology, and viability whereas molecular aspects assessed including sperm DNA fragmentation. Additional tests, such as the Fructose Test, were conducted. All data from the above performed test was gathered in excel files which later on statically analyzed by SPSS software. The study explores the relationship between various factors and their influence on semen parameters and DNA fragmentation. Correlations were observed among these factors, categorized as health, lifestyle, diet, Sexual Abstinence Period (SAP) and environmental factors. These relationships reveal how these factors may affect male reproductive health. The study found that age and BMI lowers semen quality. Lifestyle factors like tobacco, cigarette smoking, and alcohol use negatively affected DNA fragmentation but no other semen characteristics. Dietary factors, including a vegetarian diet, had some positive influence on semen quality, while caffeine consumption, fast food, food supplements, and cold drink consumption exhibited a negative impact. Medical history and stress were also found low impact to influence semen quality. Sexual frequency had a minor influence on semen quality, and Further research is needed to understand its role. The impact of environmental factors, such as pesticides and chemicals exposers were not statistically significant and requires further

research. Seasonal changes like temperature variation throughout the year strongly influence the semen quality.

The study shows that the group with multiple risk parameters demonstrated lower values across all measured semen parameters, indicating potential adverse effects on semen quality. This study fully explains how these factors affect the semen quality and semen quality affects the male fertility in Indian population scenario. The results contribute to our understanding of how these factors affect male fertility and offer recommendations for optimizing reproductive health. The study also helps clinicians to how improve the subjects' specific treatments which is ultimately responsible for converting the patient from infertile to fertile with the help of assisted reproductive technology.

Preface

The completion of this study on the Influence of various factors on functional and molecular aspects of the semen quality associated with male infertility represents a significant milestone in my academic journey. Undertaking this research has been an enlightening and rewarding experience, and it is with great pleasure that I present this thesis/dissertation.

The motivation behind this study stems from the pressing issue of infertility, which affects numerous couples worldwide. Male infertility rates have been steadily increasing, necessitating a comprehensive investigation into the various factors that contribute to this condition. By delving into the intricate interactions between socio-demographic, environmental, behavioral/lifestyle, and genetic factors, we focus to achieve valued understandings into the multifaceted landscape of male infertility.

Acknowledgement

I wish to extend my heartfelt gratitude to everyone who has contributed to the successful completion of this study. First and foremost, I would like to thank **God** for the blessing throughout the journey.

I express my deepest appreciation to my esteemed co-supervisor, **Prof. (Dr.) Jibanananda Mishra**, Dean (Prof.) RIMT University, Mandi, Govindgarh, Punjab who originally served as my supervisor during his tenure at Lovely Professional University. His unwavering guidance, profound scientific acumen, and exceptional mentorship were instrumental in shaping and conceptualizing this work. His mentorship has been a cornerstone of this work, and I am deeply grateful for his support and encouragement.

I also extend my sincere thanks to my supervisor **Dr. Ashish Aggarwal**, Associate Professor, Lovely Professional University, for his continuous support, insightful feedback, and unwavering encouragement throughout this journey. His guidance has been pivotal in refining the scope and execution of this study, and I am profoundly thankful for his patience and understanding.

I would also like to express my heartfelt gratitude to the Head of the Department, **Dr. Umesh Goutam**, Professor, Lovely Professional University whose unwavering support, insightful guidance, and leadership have been invaluable throughout my doctoral journey. My sincere thanks also go to the Head of the School, **Dr. Neeta Raj Sharma**, Sr. Dean, School of Bioengineering and Biosciences, Lovely Professional University for their constant support and for creating an academic environment that fosters learning, growth, and innovation. I am deeply grateful to **School of Bioengineering and Biosciences**, **Lovely professional university** for providing the resources, academic environment, and community that made my PhD possible.

I would like to express my profound gratitude to the staff and researchers at the Femcare Fertility Clinic, Pune, Maharashtra, with special mention to **Dr. Pallavi Tiple**, for the invaluable support. Their tireless assistance was pivotal to the completion of this study. Without their unwavering support, this research would not have been possible. I am deeply indebted to **Mrs. Gurminder Virk** and **Mr. Sarthak Gandhi**, for their

invaluable assistance during the sampling process. Their expertise, insightful discussions, and shared knowledge enriched this study and significantly enhanced its quality.

My heartfelt thanks also go to the participants of this study, whose willingness to contribute semen samples and share personal information was instrumental in advancing our understanding of male infertility. Their trust and collaboration have made a significant contribution to the progress of scientific research in this field.

I am sincerely grateful to my dear friends, **Miss Durdana Sadaf**, **Dr. Keval Nikam** and **Dr. Sagar Shelar**, for their unwavering encouragement, insightful advice, and steadfast patience throughout this academic journey. Their support has been a constant source of motivation and reassurance.

I would also like to appreciate **my parents, my children Naysha and Naksh** for their patience. A special and heartfelt acknowledgment goes to my better half, **Dr. Sneha Kale**, whose love, understanding, and unwavering support have been my greatest strength. Her encouragement has provided me with the resilience and inspiration needed to overcome challenges and bring this study to its fruition.

[Kale Sudarshan Nagorao]

Table of Contents

List of Tables

List of Figures

List of Appendices

Chapter 1

Introduction

Infertility is a potential issue in modern life that affects millions of couples worldwide. However, it is hard to diagnose and treat since it is not an issue of individuals but a disease of couples. Several factors are responsible for infertility, while infertility in men is continuously increasing every year throughout the world. Male infertility may be caused due to abnormal spermatogenesis or spermiogenesis. Stress, sleep quality, physical activity, food, and lifestyle alter the semen quality in infertile men.

1.1 Introduction to male infertility

According to a study, infertility affects approximately 60–80 million couples universal [1]. The diagnostics of couples are focused on uncovering the underlying causes of infertility since it appears to affect couples rather than individuals. Male infertility caused by spermatogenesis or spermiogenesis failure, in addition to mechanical obstructions, delays healthy spermatozoa from merging with the egg cell [2]. Assessing sperm parameters like count, motility, morphology, and increasing motile sperm count is done manually by specialized diagnosticians or using computer assisted semen analysis (CASA) methods like technology to accurately evaluate swimmer's motility in the seminal fluid. According to World Health Organization (WHO), the test of sperm parameters includes the evaluation of macro parameters and microparameters of the semen [3]. Many variables affect sperm quality, including genetics or diseases, and lifestyle factors like food, exercise, and sleep [4].

Millions of couples worldwide struggle with male infertility. Male infertility is the failure to conceive after 12 months of frequent unprotected sexual contact with a fertile spouse, according to the WHO [5]. Infertility affects around 15% of couples worldwide, with male factors accountable for infertility in nearly 50% of instances [6].
In 2021, over 50 percent of nations and provinces had fertility rates that were under the thresh old for replacement [7]. Men appear to become infertile for genetic, environmental, or lifestyle causes. These factors appear to have an impact on the functional and molecular components of sperm quality, resulting in poor fertilisation and lower pregnancy rates.

The concentration, motility, shape (see figure 1), and molecular composition of spermatozoa characterise semen quality, an important criterion in male fertility assessment [8]. Spermatozoa are extremely specialised cells that aid in fertilisation and embryonic development. Spermatozoa's molecular composition, composed of deoxyribonucleic acid (DNA), ribonucleic Acid (RNA), and proteins, appears to influence fertilisation and the growth of embryos. Several

reasons appear to have an impact on the functional as well as molecular components of semen quality, resulting in male infertility. Genetic abnormalities, environmental and lifestyle factors, in addition to comorbidities are some of the known risk factors.

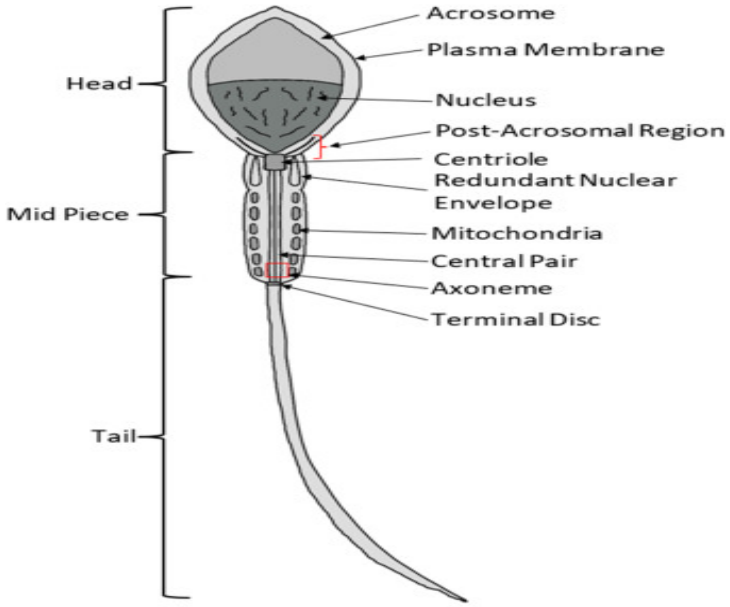


Figure 1: Structure of spermatozoa

1.1.1 Factors responsible for male infertility

Several aspects are involved in male infertility such as socio-demographic, environmental, behavioural/lifestyle factors as well as genotype or biological causes. According to the study [9], lifestyle has a greater impact on male infertility while the foremost reasons of male childlessness also include biological or physiological conditions such as azoospermia. Genetic risk factors like chromosomal abnormalities are significantly identified in addition to socio-demographic factors including age and abnormal Body Mass Index (BMI) that upsurge the danger of infertility. Figure 2 shows a few studies conducted focusing on individual risk factors affecting semen quality associated with male infertility [9].

Genetic abnormalities are a major male infertility risk, accounting for around 15-30% of infertile males [10, 11]. Genetic disorders appear to impact the count, morphology, and motility of sperm, resulting in poor fertilisation and lower pregnancy rates. Genetic problems such chromosomal abnormalities, Microdeletions of Y chromosome, and single gene mutations appears to cause male infertility [12].

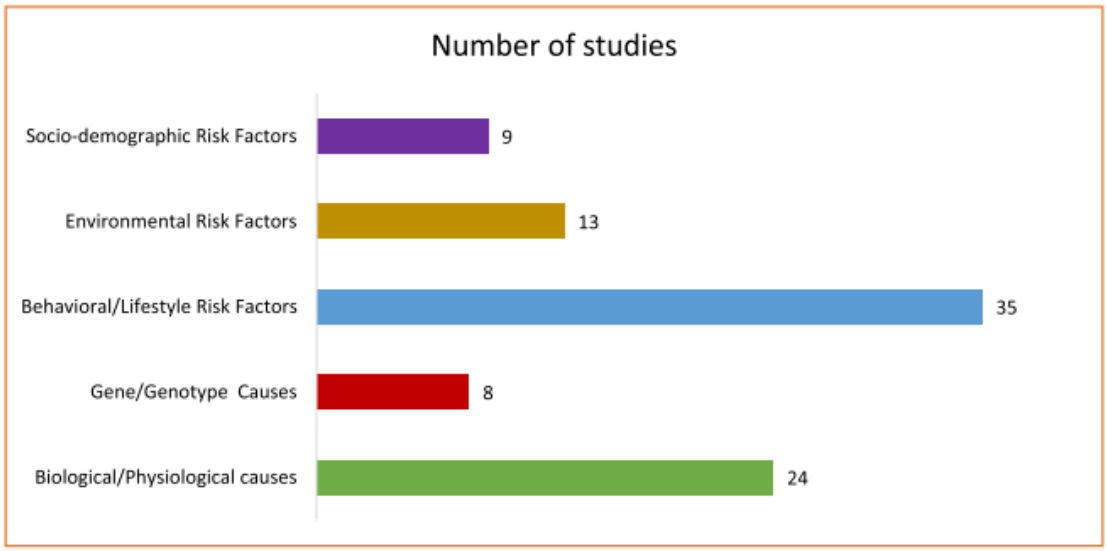


Figure 2: Major causes of male infertility

Environmental factors, such as toxicity and pollution, appears to also have effect on the functional and molecular components of sperm health. Environmental toxins and pollutants like heavy metals, pesticides, and industrial chemicals have been related to poor sperm quality and male infertility [13, 14]. Toxins and pollutants appear to harm spermatozoa by causing oxidative stress, DNA impairment, and apoptosis, resulting in poor fertilisation and lower pregnancy rates.

Diet, exercise, and smoking appears to all have an impact on the functional and molecular components of sperm quality. According to study, smoking, poor diet, and lack of exercise are related to poor sperm quality and infertility in male [15]. The environmental variables also damage spermatozoa by instigating oxidative stress, DNA damage, and apoptosis that further results in poor fertilisation with reduced pregnancy rates. Diabetes, hypertension, and obesity appears to influence the functional and molecular elements of semen quality. Comorbidities appears to harm spermatozoa by causing oxidative stress, inflammation, and hormonal disproportions, which appears to lead to poor fertilisation and lower pregnancy rates [16].

Numerous studies have explored the impact of risk factors on the functional and molecular quality of sperm in male infertility. For instance, smoking has been associated with reduced sperm count, motility, and morphology, along with increased DNA fragmentation and elevated levels of reactive oxygen species (ROS) production [17]. Similarly, research by Pan et al. highlighted that exposure to bisphenol A (BPA), a chemical commonly used in plastics, adversely affects sperm motility and causes significant DNA damage [18] . Furthermore,

Esteves et al. reported that obesity is linked to decreased sperm motility, higher DNA fragmentation, and alterations in gene expression within spermatozoa [19].

The molecular mechanisms underpinning these risk factors impacts on spermatozoa are complicated, involving numerous ways such as oxidative stress, DNA damage, apoptosis, and hormone abnormalities. Environmental contaminants and pollutants, may cause oxidative stress and DNA breaks in sperm cell, resulting in poorer fertilisation and lower pregnancy rates [20]. Similarly, lifestyle variables including poor food and lack of workout source oxidative stress and inflammation, impairing sperm function [21]. Hence, Male infertility is influenced by genetic, environmental, lifestyle, and health factors. Key causes include chromosomal abnormalities, pollution, poor diet, smoking, obesity, and stress, all of which impair sperm count, motility, and DNA integrity

1.1.2 Psychological effect of infertility on infertile couple

Infertility have far-reaching consequences, includes societal and personal harm. While advances in assisted reproductive technology (ART) like In vitro fertilization (IVF) have brought courage to many childless couples, access to treatment appears to be limited by medical coverage and affordability, leading to mental stress. The experience of infertility appears to also have profound psychological effects, including feelings of distress, loss of control, stigmatization, and disruption during medicalization [22]. These effects appears to lead to increased anxiety and sexual dysfunction [23], as well as conflicts within relationships due to pressure to make medical decisions. Infertile couples may also experience considerable stress and emotional loss [24], with feelings of anxiety, disappointment, and rejection. Psychological effects include anxiety and relationship strain.

Legal awareness of infertility has grown. Couples appear to take family and medical leave to care for a child, parent, or spouse, or for infertility treatments such diagnostic tests, surgery, or depression therapy. Thus, infertility is now a disability [25]. Up to 20% of U.S. infertile couples are suffering from unexplained cause [26]. where problems may exist but existing technologies appears to not identify them. However, Increasing research suggests epigenetic changes in sperm may be partly responsible [27]. Diagnostic tests commonly used for male partners include semen analysis. Hence, Infertility has wide-ranging social and personal impacts, often leading to emotional distress, stigma, and strained relationships. Although assisted reproductive technologies (ART) such as IVF have given hope to many couples,

limited access due to high costs and insufficient medical coverage adds financial and psychological stress. Many infertile individuals experience anxiety, loss of control, and sexual dysfunction, while couples may face tension over medical decisions.

1.1.3 Primary tests to assess the male infertility

The major steps in the valuation of male infertility are medical history and semen quality analyses. Semen assessment is an important laboratory test that measures the quality and quantity of semen using various physical and microscopic parameters. Physical parameters, including volume and viscosity, are analysed along with other parameters such as colour and pH, which should be whitish-grey or pearly white and slightly alkaline, respectively. At the other end, microscopic characteristics include sperm concentration, motility, morphology, as well as viability [28]. In addition, tests appear to be conducted for the presence of substances like antibodies, infections, etc., that appears to affect fertility. As shown in figure3, Semen made up secretions of different glands associated with male reproduction [29] [30]. The reproductive system of males is made up of primary, secondary, and accessory components that aid in effective reproduction and produce semen [31].

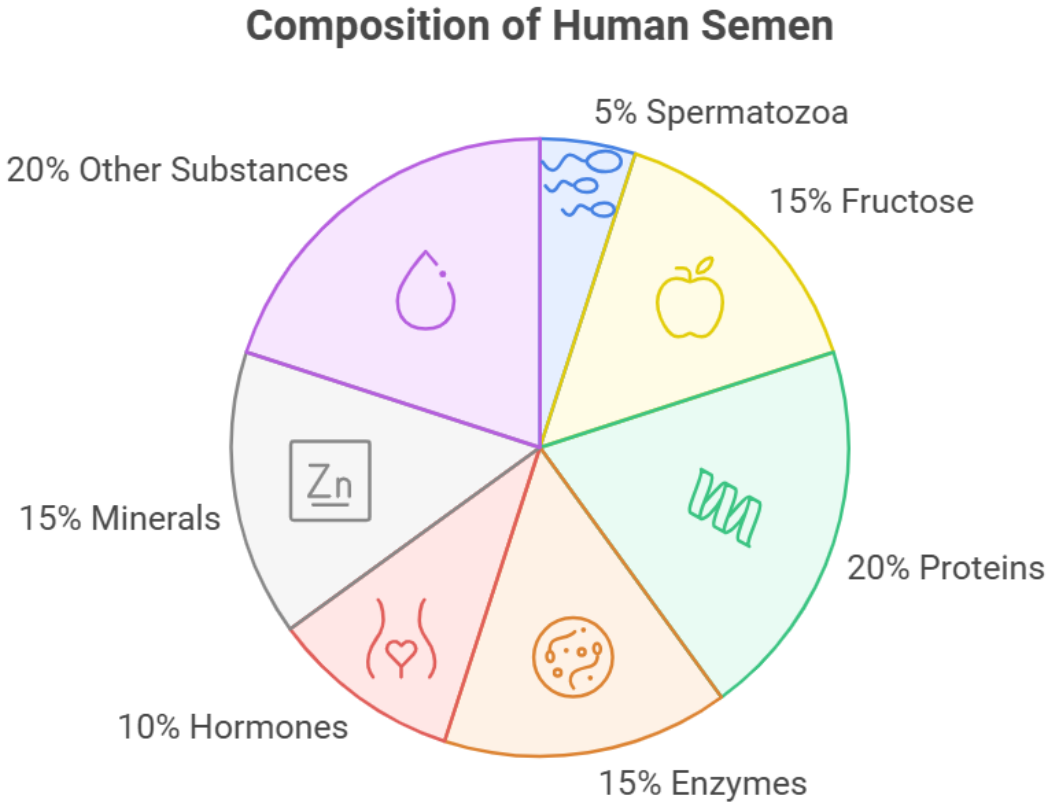


Figure 3 : Composition of human semen

Sperm count reflects the amount of sperm in the semen, and sperm movement is immotile, gradually motile, or non-progressively motile. Morphology shows abnormal sperm based on the shape and size of spermatozoa, and the percentage of live sperm in the semen is represented by viability. Thus, these parameters help detect the underlying source of male infertility that is useful in treatment decisions and predicting the success of ART. Infertility may occur when sperm are unable to fertilize an egg due to factors such as low sperm count, reduced motility, and abnormal morphology.

The Sperm Chromatin Dispersion (SCD) test plays a critical role as it provides insights beyond the conventional parameters assessed in a standard semen analysis. The Fructose test measures the fructose concentration in semen, produced by the seminal vesicles, which serves as an energy source for spermatozoa. Reduced fructose levels may indicate dysfunction in the seminal vesicles or blockage in the ejaculatory ducts. The SCD test evaluates DNA fragmentation, reflecting sperm DNA integrity. Elevated DNA fragmentation levels appear to lead to infertility, increased risk of miscarriage, and potential genetic abnormalities in offspring. This test helps identify individuals with high DNA fragmentation levels who may benefit from targeted interventions, such as antioxidant therapies or advanced sperm selection techniques [32].

The standard sperm examination, however, is insufficient to explain male infertility in more than 30% of cases [33]. Other male fertility diagnostic assessments are available, although fertility clinics rarely use them. These assessments are designed to determine sperm capacitation ability, status of acrosome, integrity of membrane, contact with the oocyte, activity of mitochondrial, integrity of DNA, and other factors [34]. More research is examining male infertility's molecular causes [35, 36]. Most often, proteomic methods compare sperm samples from fertile and infertile men to find proteins involved in infertility problems. Male infertility risk factors negatively affect sperm protein content in these studies. In addition to diseases, several variables are affecting male reproductive potential [36]. The most prevalent risk factor for infertility is age. While men are fertile throughout their lives, however fertility potential begin to drop after the age of [37]. A variety of factors appears to have an impact on fertility, such as nutrition, weight, physical activity, psychological stress, lifestyle changes, smoking, and drug or alcohol use. Furthermore, certain other factors have been identified to negatively impact semen quality, including medication, diseases such as diabetes, exposure to synthetic chemicals, clothing, sleep, and other environmental factors [33, 38]. The evaluation of male

infertility mainly involves medical history and semen analysis. Semen tests assess physical traits like volume, color, and pH, and microscopic features such as sperm count, motility, morphology, and viability. These parameters help identify issues like low sperm count or poor motility that can hinder fertilization and guide treatment decisions for infertility and ART success and advance assessment SCD will help out for diagnosis.

1.1.4 Global Market of Infertility Treatment

The figure 4 depicts the increased revenue in the global market for male infertility therapy, which indicates a rise in infertility concerns [39]. Male infertility treatment has been steadily increasing since 2014. However, the market is being propelled by various factors such as a sedentary lifestyle, increasing age, and environmental influences that have led to an increase in male infertility. The male infertility treatment sector has been steadily increasing since 2014.

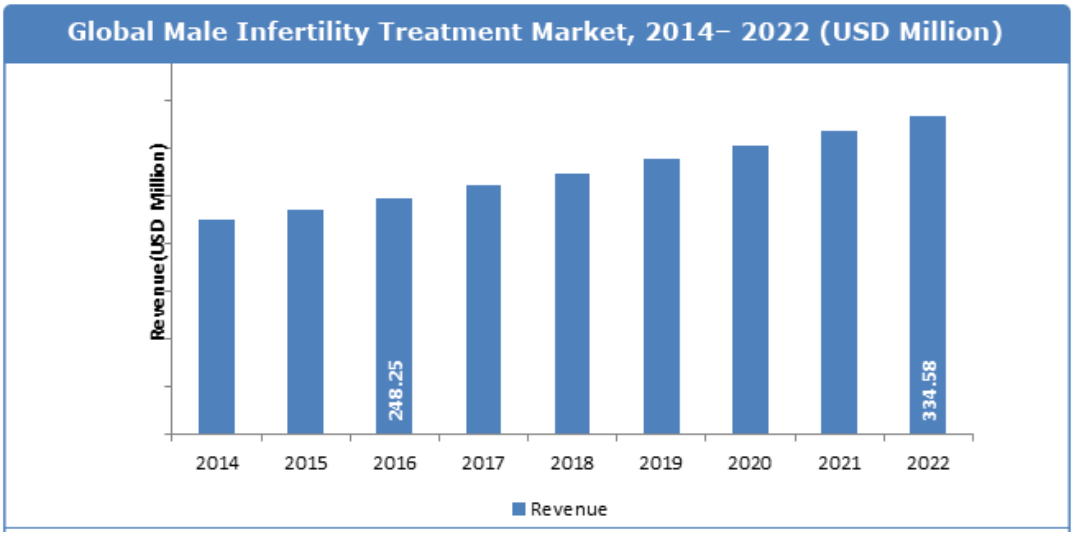


Figure 4: Global market for male infertility treatment

However, medical tourism, awareness, low-cost treatment, and healthcare technology research and development are expected to grow the industry in the next years explained in figure 4 [39]. The universal market for male infertility assessment is divided into five groups based on techniques: DNA fragmentation, oxidative stress analysis, sperm penetration, micro examination, sperm agglutination, and others [34].

Thus, male infertility affects millions of couples globally, with risk factors causing poor sperm quality and reduced pregnancy rates. Genetic problems, environmental factors, lifestyle variables, and comorbidities that affect sperm quality appears to cause male infertility.

Understanding the molecular basis behind the effects of these risk factors on spermatozoa is critical for creating effective male infertility diagnostic and treatment options.

1.2 Problem statement

Male infertility is a vast and intricate problem that affects several million couples globally has shown in figure 5 [7]. In nearly half of these cases, male influence infertility is a contributing factor. Semen quality affects male fertility and is affected by environmental, genetic, and lifestyle variables [9]. Smoking and air pollution are known to negatively affect sperm quality and molecular markers. For instance, a recent study highlighted that smoking leads to increased sperm DNA damage, along with a reduction in sperm motility and morphology. Air pollution, likewise, has been linked to lower sperm concentration, motility, and viability [40]. Diet and exercise also play a significance appears to role in sperm health. Diets high in processed and red meats, saturated fats, and sugar have been associated with reduced sperm count, motility, and morphology.

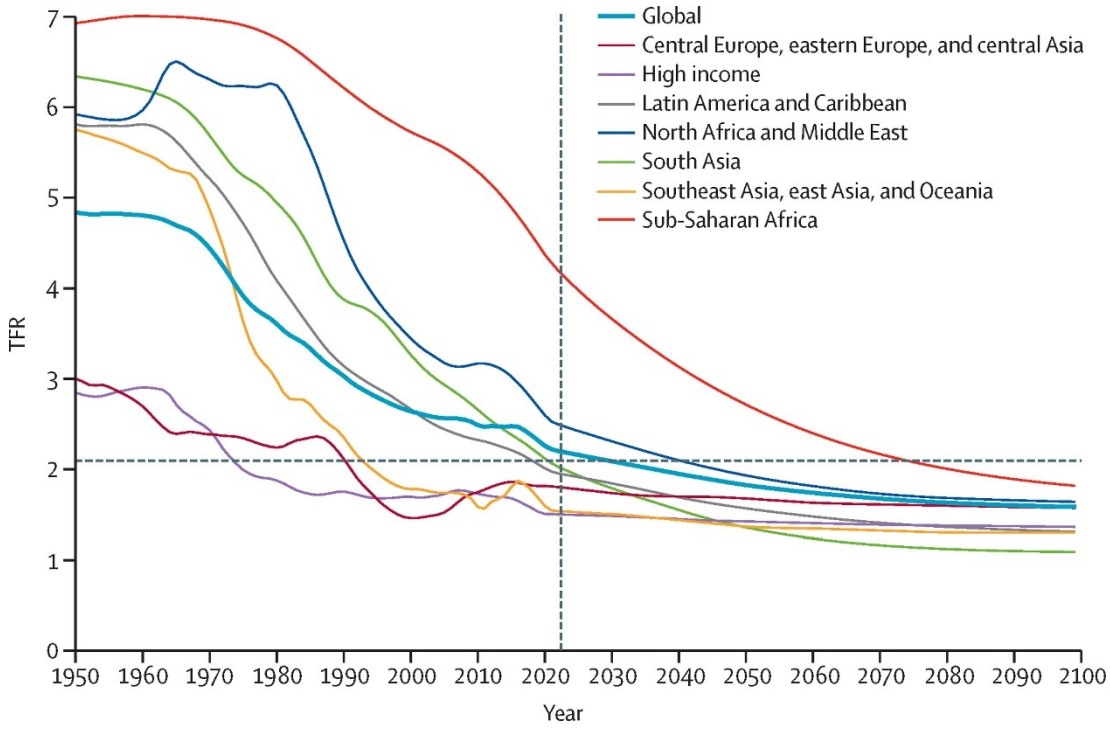


Figure 5: Total fertility rate declining globally 1950–2100

In contrast, diets rich in fruits, vegetables, whole grains, and omega-3 fatty acids are linked to improved sperm quality. Moreover, while sedentary behaviour is often associated with lower

semen quality, regular physical activity tends to enhance semen parameters [41]. In addition, there is evidence indicating these risk factors could interact with one another and with hereditary factors to reduce male fertility even further. A recent study, for example, discovered that those with high levels of perfluoroalkyl chemicals, which are widespread in the environment, had decreased sperm concentration and motility, but only in people with certain genetic variations [42]. Given the high rate of male infertility and how lifestyle and environment affect sperm quality, a study into the intricate interactions between these risk factors and male infertility is urgently needed. As a result, the proposed study seeks to assess the impact of numerous risk variables, such as age, BMI, lifestyle, and seasons on the functional and molecular components of sperm quality related to male infertility, as well as to identify potential intervention and prevention targets.

1.3 Scope of Research

Male infertility is a worldwide concern, and it is crucial to identify the risk factors that negatively impact sperm quality to develop effective interventions for improving male reproductive health. Our research aims to investigate the influence of various factors, such as age, BMI, lifestyle, and seasons on the functional and molecular aspects of semen quality associated with male infertility. The study will explore the underlying mechanisms and pathways affected by these risk factors and their contribution to male infertility.

This study aims to comprehensively investigate the multifactorial influence on semen quality with a specific focus on DNA fragmentation, using a diverse participant pool from region of Maharashtra. To date we not has observed systematically included this specific geographic population, making our study one of the first to explore semen parameter in this underrepresented demographic. By selecting participant from Maharashtra, our research brings forth unique regional insights, addressing potential environment, lifestyle and socioeconomic influence that may contribute to variation in male reproductive health.

Unlike previous studies that tend to focus on one or two factors which affecting semen parameters in isolation, our research takes a broader, integrative approach. We have analysed seventeen factors including seasonal effect as well with semen parameters. The study will comprehensively assess lifestyle factors, such as smoking, alcohol consumption, tobacco consumption, physical activity levels, and nutritional status, which are known to impact semen quality. By evaluating the interplay between these factors, we aim to build a more holistic

understanding of fertility potential. Importantly the study also included a substantial sample size (>1000), providing a robust dataset that enhances the statistical reliability and generalizability of our findings. Inclusion of large number of participants enables the detection of subtle trends and correlations that might be missed in smaller scale studies.

What sets this study further apart is its pioneering attempt to correlate DNA fragmentation with the wide range of physiological and lifestyle factors including age, BMI, smoking etc. To our knowledge, no prior research has attempted such comprehensive correlation within the context of the Maharashtra population. This integrated analysis not only contributes to scientific understanding of male infertility but also offers potential applications in personalized fertility assessment and treatment strategies. In conclusion, this research fills a significant gap in current literature by offering region specific, multifactorial, and deeply analytical insights into semen quality which influence infertility.

1.4 Role of Assisted Reproductive Technology in solving the male infertility

ART is important in resolving male infertility, which is both a personal and societal concern. While surgical techniques give an etiological approach to treating male infertility, pharmacological drugs offer both etiological and empirical therapy. Instead, ART is a symptomatic treatment for male infertility.

Alternative treatments become useful for specific subsets of males suffering from hereditary oligoasthenospermia or damage of primary testicular deprived of lumen impediment in the genital ducts, when surgical intervention such as varicocelectomy is not recommended. the use of ART or pharmaceutical stimulation of the testis to enhance spermatogenesis or epididymal sperm maturation are the mostly selected options. The decision between ART and pharmacological medicines is based on the benefits and drawbacks of each technique. Figure 6 shows ART treatment with sperm retrieval and etiological or empirical treatment categorised as pharmaceutical and surgical treatment which signifies transurethral resection of ejaculatory ducts (TURED) [43].

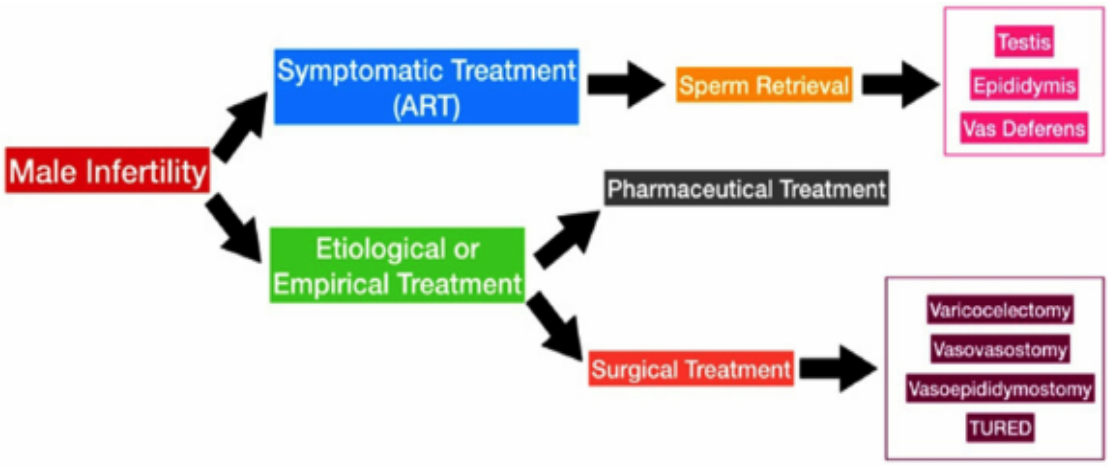


Figure 6: Types of male infertility treatment - symptomatic and etiological

Emerging research has raised concerns about the influence of ART methods like advance intracytoplasmic sperm injection (ICSI), conventional IVF, also round spermatid injection (ROSI) on imprinting abnormalities and human foetal development. These strategies have been linked to the selection of immature sperm cells missing appropriate imprinting or global methylation, which has been shown to have a deleterious impact on embryonic development [44]. Furthermore, there is evidence that ART methods may create epigenetic changes that are passed down to future generations, potentially increasing the chance of infertility and congenital defects in offspring. As a result, it is critical to concentrate on improving existing ART approaches and carefully reviewing the selection of acceptable procedures. Since epigenetic changes appears to persist throughout a person's lifespan and be handed down to future generations, enduring supplement besides health assessments of ART children are required for establishing more rigorous clinical indication [45]. While ART technologies for example ICSI and IVF have given numerous couples with infertility the chance of having biological children, it is critical to recognise the potential risks associated with abnormal epigenetic information transmission, which could lead to rising infertility rates and other health issues.

ART provides the benefit of achieving high success rates of pregnancy and live births [46]. It offers a viable option for couples facing challenges such as abnormalities in semen count, morphology, or motility. But, the utilization of ART, particularly in certain countries, appears to be financially burdensome and may pose risks to both the female partner and the descendants. Thus, it is crucial to study the possible risks related with specific procedures

within ART, such as transvaginal ultrasound-guided oocyte retrieval (TUGOR) [47]. This technique carries the inherent risk of injuring nearby pelvic vessels or organs, which appears to lead to complications such as bleeding, pelvic infections, or pelvic abscess. Additionally, complications related to anaesthesia administration are also a possibility.

Ovarian hyperstimulation, resulting from excessive pharmaceutical stimulation of the ovary, carries significant risks. Symptoms such as breathing uneasiness, discomfort, weight increase, ascites, oliguria, or thromboembolism appears to pose a life-threatening situation for the female partner. There are also concerns about the possible link between ovarian stimulation and the development of malignancies [48]. The relationship between fertility medication supervision and ovarian cancer rests highly debated and controversial [43].

1.5 Research Objectives

The objectives of the research work to evaluate the impact of various factors, including age, BMI, and lifestyle, on the functional and molecular aspects of semen quality associated with male infertility are as follows:

- Objective I:
  Semen collection and storage along with the personal data in the questionnaires
- Objective II:
  Evaluation of functional aspects of semen quality in the collected semen samples
- Objective III:
  Determination of the molecular aspects of semen samples by sperm chromatin dispersion method.
- Objective IV:
  Assessment of the impact of Age, BMI, and lifestyle factors on semen parameters

Chapter 2

Review of Literature

2.1 Background of infertility

15% of couples worldwide suffer with infertility [21]. 50% of these cases involve male infertility. Hormonal disorders, compromised spermatogenesis, genetical abnormalities, immunologic challenges, ejaculatory errors, reproductive tract infections, hyper viscous semen, and structural or anatomical defects like varicocele and obstructive issues cause most male infertility [49]. Figure 7 shows the complexity of male infertility causes. Environmental or occupational factors like smoking, opiates, pesticides, and radiation appears to also affect gamete health and male fertility. Idiopathic male infertility results when the reason of male infertility is unclear, which affects 30–40% of cases [50]. The WHO describes infertility as the inability of a sexually active couple, who are not using contraception, to achieve pregnancy within a year [51]. But despite substantial research, a sizable portion of male infertility cases still have unsolved causes as there are multifactor behind it has been shown in figure 7 [52].

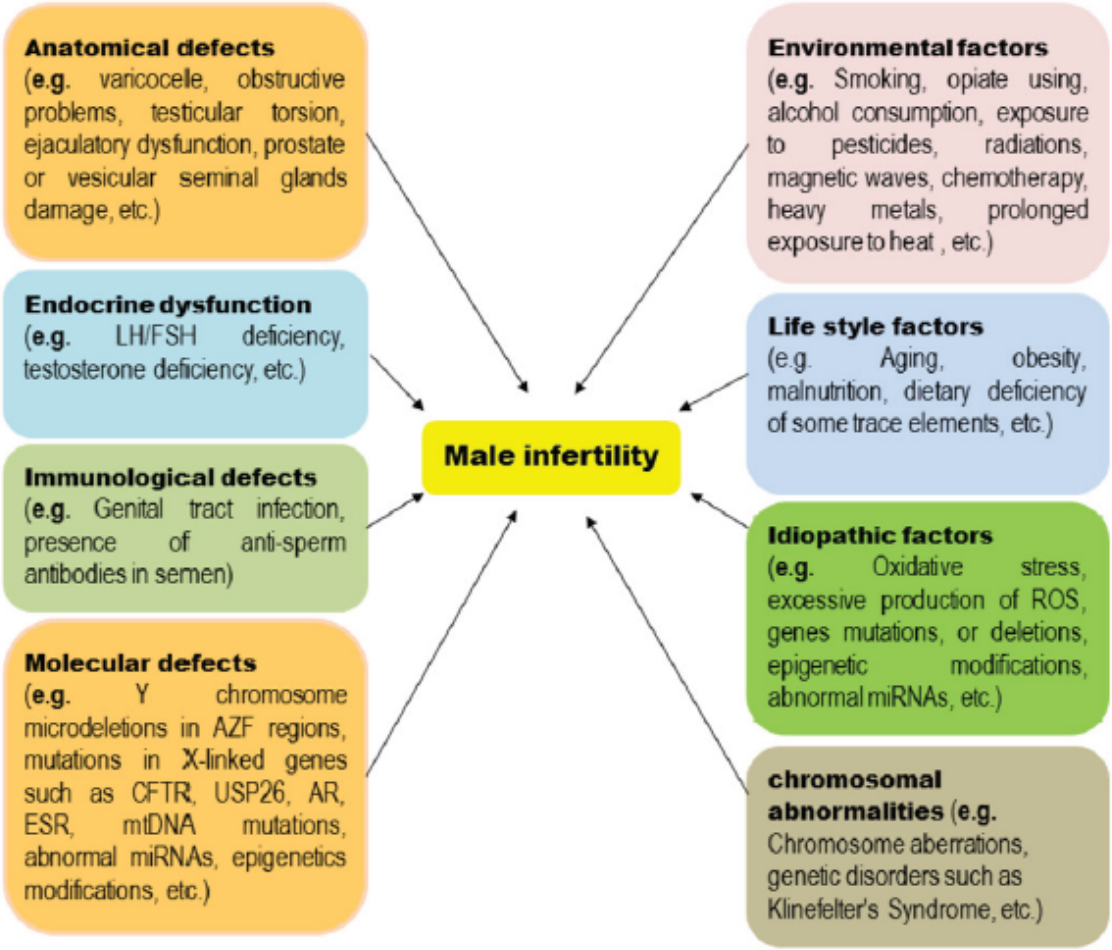


Figure 7: Primary causes of male infertility: a schematic analysis

Men experiencing infertility due to unknown causes often exhibit normal sperm parameters. A common factor contributing to this type of male infertility is oxidative stress, which results from an overproduction of reactive oxygen species (ROS) [53]. ROS appears to contribute to gene mutations, DNA fragmentation in sperm, and genetic disorders. Molecular genetic conditions, which occur in approximately 15–30% of cases, significantly impact the development of male infertility by affecting various physiological processes, such as steroidogenesis, sperm production, maturation, function, and quality [53]. Understanding the associated genes, putative regulatory mechanisms, and molecular genetic variables will help us better understand the pathophysiology of this illness.

In physical exams, genetic, endocrine, and biochemical laboratory tests, men with idiopathic male infertility get normal results. Azoospermia, oligozoospermia, teratozoospermia, and asthenozoospermia are examples of aberrant semen parameters that are frequently found in their semen analysis [54]. Idiopathic sperm abnormalities may be caused by ROS, unexplained genetic and epigenetic abnormalities, and environmental pollution-induced endocrine disruption. The testis, which plays a crucial role in spermatogenesis, expresses 74% of all human proteins (n = 19692), with 1980 of these genes having higher levels of expression in the testis than in other tissue types. The majority of these proteins play a role in spermatogenesis [55, 56].

The seminiferous tubules in the testis serve as the location for spermatogenesis, a complex process. This includes the multiplication of spermatogonia, their transformation into spermatocytes, the meiotic division of round spermatids, and the release of fully developed spermatozoa into the lumen of the testicular tubules [57]. Male infertility appears to come from mutations in any one of these genes that cause problems in spermatogenesis. It is difficult to identify the genetic reasons of male infertility because these genetic flaws are frequently brought on by de novo mutations during gametogenesis and are typically not heritable due to the infertile phenotype. The genetic and molecular mechanisms behind various male infertility phenotypes, such as azoospermia, oligozoospermia, teratozoospermia, and asthenozoospermia, have been the subject of several investigations despite these obstacles.

Particularly in cultures where having children is highly prized, infertility appears to be a difficult condition with psychological, physiological, and financial repercussions. Failure to become pregnant after 12 months of unsafe sexual activity is infertility [58]. which is designated as a reproductive system disorder by the international committee monitoring

assisted reproductive technologies (ICMART) of the WHO. There are two types of infertility: primary infertility, in which the male has never been able to become pregnant with a woman, and secondary infertility, in which the guy has been able to become pregnant with a woman in the past but is currently unable to do so. Male infertility, often referred to as subfertility, appears to vary in severity, ranging from partial to complete infertility. It may arise from issues such as irregular sperm shape (teratozoospermia), reduced sperm movement (asthenozoospermia), lower sperm vitality (normozoospermia), a decrease in sperm count (oligozoospermia), or a combination of these factors. Most subfertility instances are brought on by intrinsic testicular problems. Infertility and population expansion are two fundamental obstacles to human reproduction. Rapid population growth is occurring in developing nations like India, China, and Bangladesh, which has reduced agricultural production and per capita income. With an estimated 6% rate in the UK, 10% in the USA, and 15.7% in Denmark, infertility is a problem that affects people all over the world [59]. Infertility rates appear to reach 30% or higher in some nations, including Nigeria, the Republic of Sudan, and Cameroon. Primary and secondary infertility rates in south-eastern Nigeria are, according to studies, 65% and 35%, respectively. The WHO task committee on the diagnosis and treatment of infertility estimates that 15% of the world's population suffers from infertility [60]. 10% of couples, on average, have trouble starting a family, which might make them feel unsuccessful. The wife is customarily held responsible for infertility in India, where cultural, religious, and social traditions make bearing children all but necessary. The main reasons and risk factors for male infertility, including environmental and behavioural variables, are covered in this essay along with treatment alternatives.

Approximately 7% of men globally are infertile [61]. Congenital deformities, hormone imbalances, genetic abnormalities, behavioural decisions, iatrogenic therapies, environmental contaminants, and lifestyle variables are only a few of the causes of this illness [62]. Due to the pervasiveness of environmental pollutants, environmental pollution has emerged as a significant factor in the rising prevalence of male infertility worldwide. Recent studies have demonstrated that sperm quality and human fertility are significantly impacted by air pollution [63]. Male fertility is greatly influenced by the quality of the sperm, and environmental pollution appears to harm the processes of spermatogenesis, steroidogenesis, Sertoli cell development, and sperm function, reducing male fertility [20]. Additionally, many organic and inorganic pollutants are discharged into the environment every day, and they may affect human fertility. Although environmental chemicals such as industrial waste, pesticides, insecticides,

herbicides, food additives, etc. have a negative impact on spermatogenesis in adult men, there is a lack of information on how these chemicals directly affect the general population because the studies that are currently available are typically carried out in occupational settings where the population is exposed to high concentrations of these substances [64].

2.1.1 Indian scenario of infertility

Indian scenario of infertility: As per recent survey of government of India Total fertility rate decreasing year by year. TFR is the number of children that women have in her whole life. TFR is very important for knowing the population trends of any country. As per the figure 8 data, TFR of India is continuously declining from 1880 to till the date [65]. In the year 1880 average TFR of India was 5.7, eventually it dropped consistently and in 2020 it was 2.2. Figure 9 shows TFR was varied for Urban region and Rural region of country and it was 1.6, 2.2 subsequently [66]. It has been observed that Maximum states of India have lower TFR than the standard replacement level 2.1, Bihar has highest TFR whereas Sikkim has lowest TFR rate. As per recent study India's TFR may fall down to 1.29 till the 2050 [67].

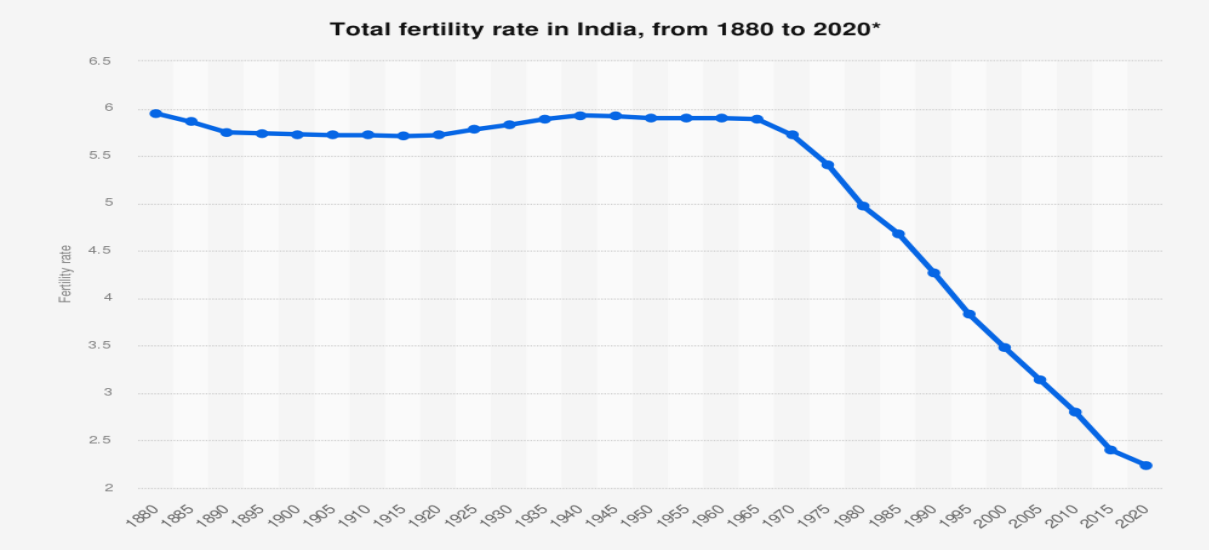


Figure 8: Total fertility rate in India, from 1880 to 2020

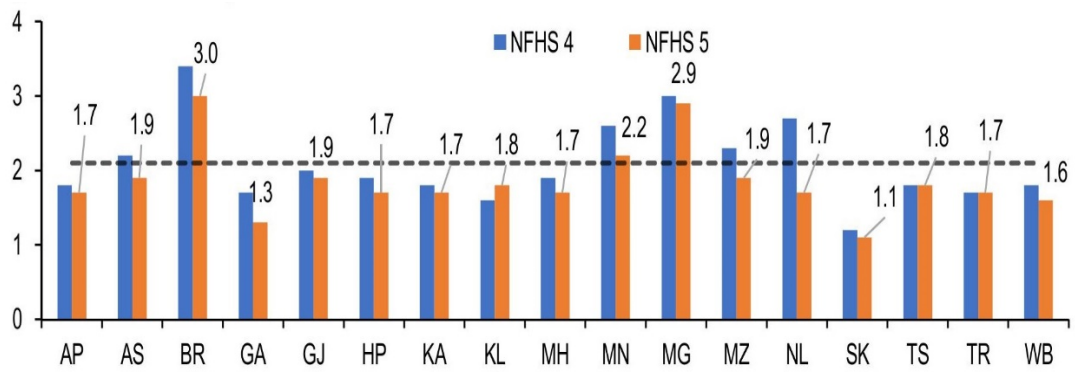


Figure 9: Total fertility rate across the states of India

Note: AP = Andhra Pradesh, AS = Assam, BR = Bihar, GA = Goa, GJ = Gujarat, HP = Himachal Pradesh, KA = Karnataka, KL = Kerala, MH = Maharashtra, MG = Meghalaya, MZ = Mizoram, NL = Nagaland, SK = Sikkim, TS = Telangana, TR = Tripura, WB = West Bengal

2.1.2 Global status of infertility

Millions of couples worldwide struggle with male infertility. An analysis of recent literature found that 48.5 million couples globally suffer from infertility [68]. We found that global male infertility rates range from 2.5% to 12%. Given the paucity of actual rates of infertility, these rates are based on self-reported statistics and range widely. Because of the widespread effects of male infertility, more study is needed to determine its origins and potential remedies.

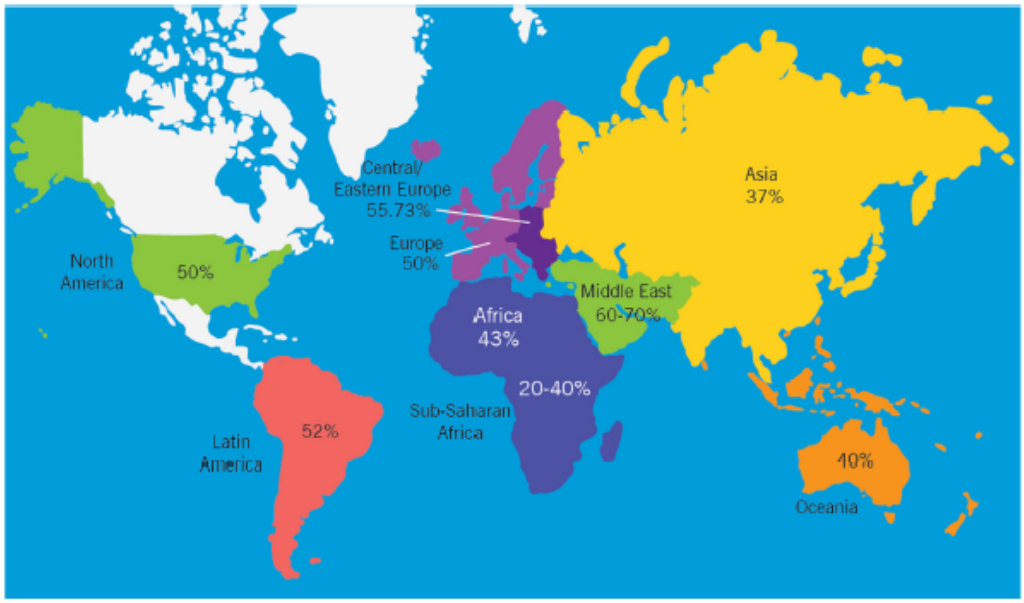


Figure 10 : World map showing male-factor infertility rates per area

Figure10 depicts the percentage of male factor-related infertility in the United States, South America, Europe, Africa, Central/Eastern Europe, the Middle East, Asia, and Oceania [69]. Globally, there is a problem with male infertility, albeit rates vary by area. Comparatively to less developed nations, developed nations like North America, Europe, and Australia record infertility rates with greater accuracy. Australia has 8% infertile men, compared with 4.5% to 6% in North America, while 9% of men over 40 have gone to an infertility clinic.

Table 1: A Comprehensive Analysis of Male Infertility: Insights from Global Studies on Male and Female Infertility

Region	**Males reported infertile**	**Couples reported infertile**	**Multiple-factor couples with men**	**References**
North America.	4.5-6%	15%	50%	[54]
Middle East.	Unidentified	Unidentified	60%-70%b	[58]
Sub-Saharan Africa	2.5%-4.8%	12.5%-16%	20-40%	[59]
Europe	7.5%	15%	50% of all infertile couples	[45]
Australia	8%; 9%b	15%	40%	[60]
Central/Eastern Europe	8%-12%	20%	56%	[61]
Asia	--------	--------	37%	[62]
Latin America	--------	--------	52%	[62]
Africa	--------	--------	43%	[62]

European rates are 7.5% (Table 1) and are cross-checked against data from the National Health Statistics Report (NHSR) by the CDC National Health Interview Survey, the Australian Institute for Health and Welfare (AIHW), and the infertility standards for men set by the European Association of Urology (EAU) [54]. Figure 11 estimates were determined using the estimate that a single male factor contribute for 20–30% of total infertility patients [69].

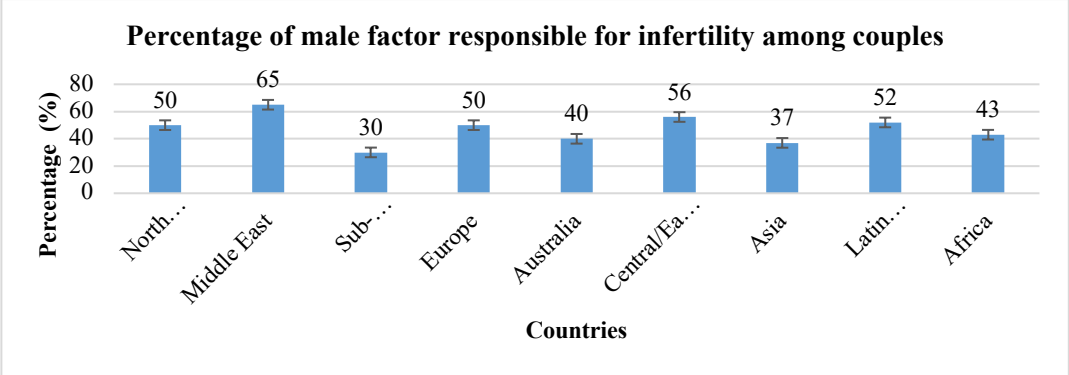


Figure 11 : Graph showing percentage of male factor responsible for infertility among couples

Recent years have seen advances in stem cell-based, the next-generation male reproductive therapy. Using embryonic, induced pluripotent, and glioblastoma stem cells, many *in vitro* procedures and organ modeling have been developed to generate sperm cells [70]. Human induced pluripotent stem cells (hiPSCs) have been studied for treating male infertility [71]. Using clustered regularly interspaced short palindromic repeats (CRISPR-Cas9) gene editing, hiPSCs have the potential to restore spermatogenesis and treat genetic diseases. Exosomes made from hiPSCs may also be used therapeutically to help individuals who have undergone radiotherapy or chemotherapy regain their ability to produce sperm. New avenues for treating male infertility have also been made possible by the spermatogonia stem cells' capacity for regeneration and self-renewal. Cryobank spermatogonia tissue autografting has been suggested as a method for paediatric kids who have had gonadotoxic therapy to preserve their fertility [72]. Before stem cell therapy appears to manage and treat male infertility, ethical difficulties, and the risk of inherited harm from *in vitro* stem cell production must be overcome.

2.2 Factor causes of Infertility

Lifestyle factors like smoking, alcohol, recreational substance abuse, becoming overweight, and psychological stress appears to induce male infertility [73]. These risks might also include occupational or environmental exposure to harmful substances. Congenital, acquired, and idiopathic causes and risk factors appears to all be categorised as contributing to the rising prevalence of male infertility [74]. Genetic factors contributing to male infertility include chromosomal abnormalities that impair testicular function, Kallmann syndrome, congenital absence of the vas deferens associated with cystic fibrosis gene mutations, and Y chromosome microdeletions causing isolated spermatogenic defects. Among the acquired causes, varicocele is the most common and treatable, accounting for 40% of cases [75]. Approximately 30–50%

of cases of male infertility are with unknown cause, meaning they have no known aetiology or contributing female infertility. With idiopathic male infertility, which includes changed semen properties and oxidative stress, roughly 37 million men are affected by male oxidative stress infertility.

2.2.1 Human Age

As more men want children at some point in life, male getting older and fertility are becoming public health issues. The basal membrane, seminiferous tubules, and tunica albuginea of the testis all suffer from advanced age. Lipofuscin, a "ageing" pigment, accumulates in Leydig cells, which also decline in number [76], Reduced dark type and intratubular clustering of pale type spermatogonia are two age-related alterations in spermatogenesis. At the spermatocyte I stage, spermatogenesis appears to be stopped, and many spermatid abnormalities have been seen [77]. Sharma's research showed that sperm motility and vitality decline with age, with the effect on sperm count being the least significant . From the age of 20 and 30, sperm parameters were normal, but beyond the age of 35, they declined dramatically [78].

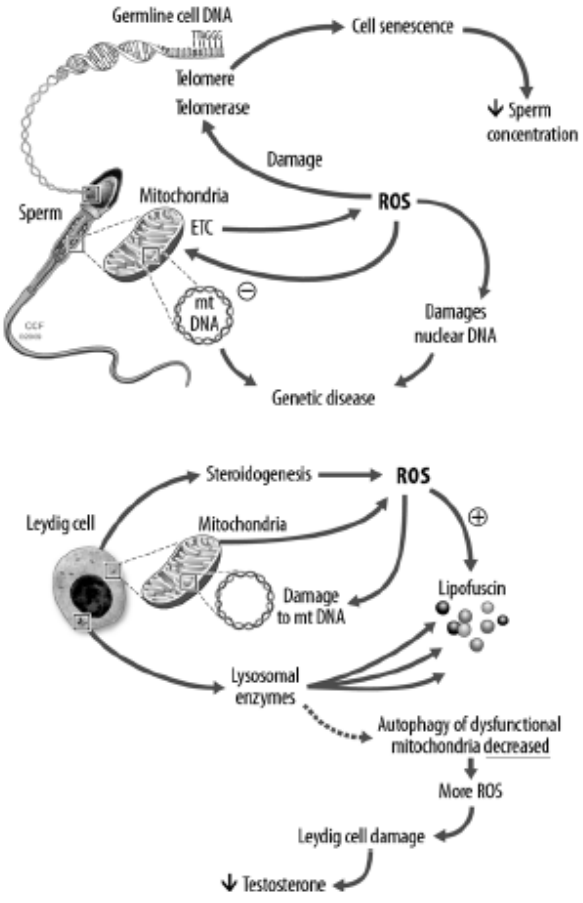


Figure 12: Constant sperm generation of reactive oxygen species

Throughout the past decade, damage to DNA in sperm and aging have been extensively studied. Singh et al. observed a developed incidence of duel-stranded DNA breaks in age-old men using the comet DFI assay, while Wyrobek et al [79], reported a contrary relationship between male age and the DNA fragmentation index (DFI) using the sperm chromatin structure assay. It is suggested that DNA damage increases with age, potentially due to oxidative stress. In older men who experience repeated failures with ART, oxidative stress-induced DNA damage may be a contributing factor. It has been proposed that DNA damage increases with age, possibly due to oxidative stress. For older men who face repeated failures in ART, oxidative stress-induced DNA damage could be a contributing factor. Additionally, age may influence single-strand DNA fragmentation. While oocytes appear to repair single-strand breaks, defective repair mechanisms may result in poor or unsuccessful blastocyst development. Consequently, figure12 shows oxidative stress-related DNA damage could lead to various genetic abnormalities [80, 81].

Plas et al. examined the link between paternal age and chromosomal abnormalities. They found that in men over 45, the incidence of structural chromosomal anomalies was four times greater than in those aged 20–24. This increase could be attributed to the greater frequency of germ cell mitoses and meiosis as men age. Furthermore, children of older fathers may have a 20% higher likelihood of inheriting autosomal dominant diseases. Paternal age also impacts offspring development [82]. Reichenberg et al. reported that children born to fathers aged 40 or older had a 5.75 times greater chance of developing autism spectrum disorder compared to those with fathers under 30, suggesting that paternal age appears to influence cognitive and learning abilities in offspring [83].

De novo mutations and changes in genomic imprinting were cited as potential reasons. According to earlier studies, there is a higher chance of trisomy in a man's offspring who is older than 41 years old [84]. Given the strong association between spontaneous mutation rate and paternal age, it is conceivable that imprinting disorders like Beckwith-Wiedemann syndrome may be more common in children of older dads. This condition, which includes macroglossia, abnormalities in the abdominal wall, and a propensity for embryonic tumours, is brought on by the paternally expressed gene for insulin-like growth factor [85]. Therefore, as men age, fertility declines due to testicular tissue damage and reduced spermatogenesis. Sperm motility, vitality, and DNA integrity decrease after age 35, mainly from oxidative stress.

Older paternal age is linked to higher risks of chromosomal abnormalities, genetic mutations, and disorders such as autism and Beckwith-Wiedemann syndrome in offspring.

2.2.2 lifestyle factors

Figure 13 shows modifiable lifestyle choices, also referred to as lifestyle factors, have a big impact on fertility as well as general health and wellbeing. There is evidence to show that certain reproductive and genetic disorders may be linked to rising paternal age and occupation. Food intake, physical activity, managing stress, smoking, illegal and prescription drug use, consumption of alcohol, and intake of caffeine may all affect male fertility. The age at which a family is established is another crucial factor [86].

2.2.2.1 Alcohol consumption

Alcohol use was shown to be significantly associated with lower semen volume, but not with sperm parameters, according to a meta-analysis of 29,914 male participants. The volume of the sperm and its shape, however, are both negatively impacted by alcohol intake, according to a more recent meta-analysis of 16,395 males [87]. Exposure of sperm to alcohol has been shown to negatively affect sperm motility and morphology in a dose-dependent manner, with concentrations similar to those found in the serum after moderate to heavy drinking. Alcohol seems to disrupt the hypothalamus-pituitary-gonadal (HPG) axis throughout various stages of male reproduction. It appears to influence the levels of Gonadotropin releasing hormone (GnRH), Follicle stimulating hormone (FSH), Luteinizing hormone (LH), and Testosterone, as well as the function of Leydig and Sertoli cells. Consequently, alcohol may interfere with the development, maturation, and production of sperm [73].

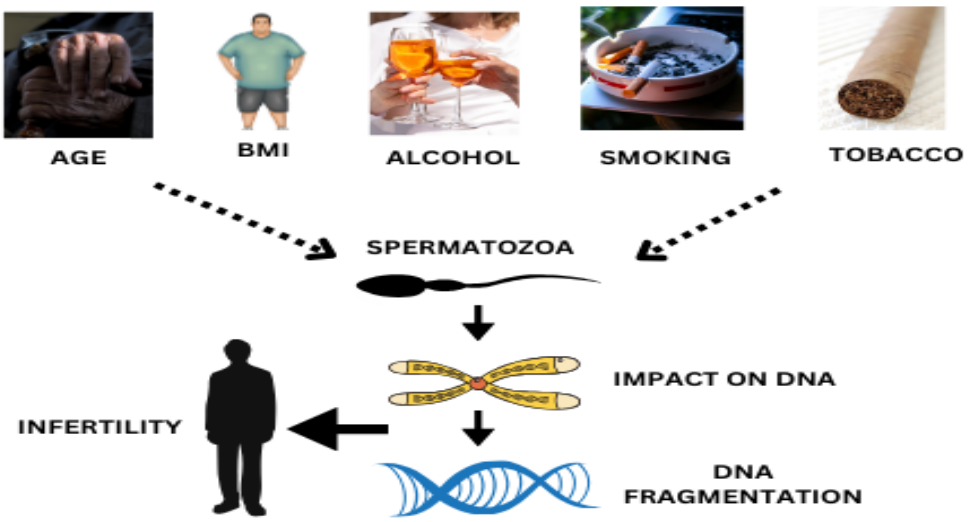


Figure 13: Lifestyle factors affect male fertility semen quality

With greater alcohol use, spermatogenesis seems to gradually reduce. Comparatively to non-drinkers, heavy drinkers were more likely to experience Sertoli cell-only syndrome and partial or total spermatogenic stoppage. Prolonged alcohol usage degrades male hormones for reproduction and semen. Studies of 8,344 healthy male volunteers found that moderate alcohol use raised testosterone but not quality of semen [88]. It has been demonstrated that chronic ethanol administration decreases the action of the steroidogenic and antioxidant enzymes in the testicles, which increases oxidative stress [89]. This might interfere with the production of testosterone and harm fertility.

In an investigation of male partners in primary infertility couples, 63% and 72% of moderate (40-80 g/day) and heavy (>80 g/day) drinkers had teratozoospermia. Neither of the heavy drinkers had normozoospermia, and the majority (64%) had oligozoospermia, which shows that testicular damage progresses as daily alcohol use increases [90]. In comparison to fertile controls, a different study found that males with severe oligozoospermia and non-obstructive azoospermia drank significantly more alcohol [91]. While the amount of alcohol taken affects how it affects male reproductive function, it is yet unknown at what point the risk of male infertility starts to increase. Furthermore, while drinking alcohol or smoking cigarettes did not have a deleterious influence on sperm parameters on their own, the two factors combined appear to have an additive effect that may have a negative impact [92].

2.2.2.2 Smoking habits

Smoking has harmful effects on sperm function, as well as negative health effects, as reported by WHO, affecting about 1.3 billion individuals or approximately 20% of the world's population, with 37% of males aged 15 or older being smokers [93]. Smoking has negative impact on spermatozoa including, capacitation, nuclear and acrosomal maturity, motility, shape, numbers, variability, and semen volume. These effects are attributed to smoke components that increase oxidative stress and inflammation and alter spermatogenesis, hypothalamic-pituitary axis function, and testicular endocrine function [93]. A global proteome strategy was used to compare 20 non-smoking normospermia males with 20 smoking patients (who smoked a minimum of 10 cigarettes per day) in the only study of its kind to date [94]. Males having a clinical history that could have caused testicular changes were excluded from the investigation[95]. Label-free LC-MS/MS was utilized to contrast 20 normospermia males that were not smokers with 20 smoking patients (who smoked a minimum of 10 cigarettes per day) in a global proteome study to determine how smoking affects human sperm. The

researchers identified 25 proteins that were differentially expressed in smokers, with one absent, 18 down-regulated, and six up-regulated. The functional analysis indicated that smokers had increased levels of proteins associated with immune and inflammatory responses [94].

S100A9 protein, an inflammation biomarker, appears to predict smokers. The scientists proposed that the reduced mitochondrial function and acrosome integrity, along with the amplified DNA damage detected in the spermatozoa of cigarette smoker, may be caused by inflammation of the accessory glands and testes [94]. Mice that were uncovered to cigarette smoke every day were used in a different proteome profiling investigation. Spermatozoa that had been isolated from the semen of unexposed and exposed mice using the swim-up method were examined by Chen et al. They discovered 22 differentially expressed proteins with 2D-PAGE and MALDI-TOF-MS; 10 of these proteins were up-regulated and 12 were down-regulated in exposed animals [96]. Western blot tests confirmed that four of the five proteins varied in their quantity. The potential drawback of inducing data obtained in rodent models to humans was emphasized as western blot examinations of spermatozoa from smoking men did not reveal the same pattern, possibly due to different testing circumstances [96]. The deregulated proteins in mice were mainly categorized into biological regulation, cellular, developmental processes, localization, and metabolic processes by the GO analysis [96].

Numerous research has examined how smoking affects sperm proteins that cause distinct responses within cells in smokers. This research examined the level of expression or activity of these proteins. In one study [97], smoking decreased the activity of three antioxidant enzymes in seminal plasma: glutathione-S-transferase (GST), catalase, and superoxide dismutase (SOD) [98]. A reverse result was discovered in a novel study that used pure spermatozoa, with smokers. Three studies have found that smoking men have reduced levels of the protease acrosin (ACR) present in the sperm acrosome [99]. This decrease is linked to acrosome response inducibility.

In a research by Kumosani et al. it was revealed that males who smoked had lower plasma membrane Ca^{2+}-ATPase (ATP2B4) action in their spermatozoa [100]. The lower motility seen in the spermatozoa of smokers was considered to be caused by this drop, which was linked to higher cadmium contents in seminal plasma [100]. Smoking has been linked to lower expression of checkpoint kinase 1 (CHK1), sperm DNA fragmentation, decreased motile sperm, and creatine kinase (CK) activity. CHK1 is involved in DNA repair and cell cycle

regulation, and its decrease in smokers' sperm may contribute to DNA damage. The decrease in CK activity may disrupt sperm energy homeostasis and lead to decreased motility [97, 99].

Although CK and sperm motility showed a favorable connection, the relationship was not statistically significant [101]. Additionally, smoking was observed to raise the sperm's levels of the apoptotic markers Fas and CASP3 [102]. A list of 54 proteins was created by applying particular criteria to the fold variations of proteins deregulated in smokers. ACR, SOD, and CAT were discovered among them in three, four, and two separate studies, respectively. It is important to note that rather than through extensive proteome research, these proteins were discovered using focused identification techniques [97, 99]. Acrosomal enzyme ACR is crucial to acrosome reactions and zona pellucida invasion [103]. Men with idiopathic infertility were revealed to have lower levels of acrosin activity than men who were fertile, leading to the suggestion that it serves as a marker of fertility [104]. Since smokers' spermatozoa have lower ACR activity, tobacco usage may cause reproductive issues. Since smokers' spermatozoa have lower ACR activity, tobacco usage may cause reproductive issues.

Smoking reduced SOD and CAT activity in men's seminal plasma, which may damage sperm by causing lipid peroxidation [105]. The proteins found in humans that were subjected to gene ontology analysis showed a high enrichment in reactions to drugs, immunological responses, oxidative stress, apoptosis, and cell differentiation. A single mouse study identified oxidation-reduction, metabolism of energy, and cytoskeleton-related proteins elevated [96]. Several investigations have publicized that oxidative stress in sperm appears to be a crucial mechanism associated to smoking in both people and rats [97].

Both males and females are adversely affected by tobacco usage when it comes to reproduction. Benzo[a]pyrene (BaP), a strong carcinogen, is one of the dangerous substances found in cigarette smoke. It appears to interact with DNA and lead to mutations, which might make both men and women infertile. Smokers may have lower estrogen and progesterone levels, irregular menstrual cycles, delayed ovulation due to inadequate LH surge, prolonged conception periods, and an increased probability of premature delivery, prenatal hemorrhage, and low-weight newborns during IVF. They might also experience menopause sooner. Male smokers have been found to have reduced testosterone levels, less sperm, impaired motility, a larger percentage of defective sperm, and lower sperm counts. Asthma and congenital problems in their offspring may result from this. The chance of children developing cancer may rise in families where the

father smokes heavily. According to one study, heavy smokers' sperm counts were 19% lower than non-smokers' [106].

One more research by Ji et al. exposed that the quantity of cigarettes the father smoked before the mother became pregnant increased the likelihood that a kid would acquire cancer by the age of five. Malondialdehyde (MDA) levels are higher, motility is decreased, and acrosome response is observed in spermatozoa exposed to the seminal plasma of smokers [107]. Waylen et al.'s meta-analysis found that smoking lowers ICSI success rates for both smoking and non-smokers (22% vs. 38%) [108]. In contrast, Rybar et al. were unable to identify a connection among smoking and sperm health in any of the groups they looked into [109]. Males who drink too much alcohol run the risk of developing impotence as well as having unfavorable impact on spermatogenesis and/or sperm physiology. According to reports, 75% of fathers of children with fetal alcohol syndrome were alcoholics. The impacts of father alcohol consumption on offspring development and behavior may partially reverse after cessation, however this is unclear. Alcohol may lower cytosine methyltransferase mRNA stages, changing genomic imprinting and expressing usually silent paternal genes [110].

Smoking harms male fertility by damaging sperm count, motility, shape, and DNA through oxidative stress and inflammation. Proteomic studies show altered protein expression and reduced antioxidant enzymes in smokers’ sperm, leading to impaired energy balance and acrosome function. Smoking also lowers testosterone, increases sperm defects, and raises the risk of infertility, birth defects, and childhood diseases. Excessive alcohol use similarly disrupts spermatogenesis and may cause genetic imprinting changes affecting offspring

2.2.2.3 Uses of recreational drugs

Marijuana, cocaine, androgenic anabolic steroid (AAS), opiates, and methamphetamines appears to harm male fertility. These drugs may affect the hypothalamus-pituitary-gonad (HPG) axis, reproductive architecture, and sperm function, reducing fertility [111]. Particularly when mixed with other recreational drugs, regular use of marijuana, the most popular illicit substance among males globally, has been linked to lessen sperm number and total sperm concentration. This effect may be caused by endogenous cannabinoid system deregulation, which decreases sperm count, motility, and spermatogenesis. Competing among marijuana's Phyto cannabinoids and endocannabinoids for cannabinoid and vanilloid receptors may disrupt the endocannabinoid system (ECS) homeostasis, affecting male fertility [112].

Associated to sperm from fertile men, the endocannabinoid metabolism of infertile men's sperm is significantly altered, resulting in decreased cannabinoid production. In animal experiments, cocaine, a potent and addictive stimulant substance, has been proven to negatively impact male fertility. Prior to mating, male rats given large amounts of cocaine had reduced pregnancy rates and smaller progeny at delivery. The interruption of spermatogenesis and harm to the testicular ultrastructure, possibly brought on by cocaine-induced apoptosis, have been related to both acute and chronic cocaine exposure. For more than five years, cocaine consumption has been linked to lower sperm concentration, motility, and deviation from normal morphology. Infertile men who use cocaine are more likely to also smoke, abuse drugs, and have a history of STDs [113].

Males frequently use a class of hormones called AAS, which are derived from testosterone, to improve their physical appearance and athletic performance [114]. An increase in anabolic steroid-induced hypogonadism (ASIH) has been linked to the usage of AAS in young men and teenagers who are not professional sportsmen. In fact, a retrospective analysis of males seeking treatment for hypogonadism revealed that ASIH was the most frequent cause of severe hypogonadism (testosterone levels < 50 ng/dL). Excessive amounts of exogenous testosterone brought on using AAS have a detrimental outcome on the HPG axis and result in reversible spermatogenesis suppression, testicular atrophy, and infertility. This may result in transitory azoospermia, which may have a two-year recovery time. Furthermore, libido loss and erectile dysfunction might result from AAS-induced hypogonadism [115]. The effects of using an AAS and how the consequent hypogonadotropic hypogonadism is treated depend on the AAS's dosage, duration, and kind. According to certain research, taking steroids may permanently disturb a man's capacity to conceive, hence using AAS should be severely avoided.

Hence, recreational drugs like marijuana, cocaine, anabolic steroids, opiates, and methamphetamines impair male fertility by disrupting the hypothalamic-pituitary-gonadal axis and damaging sperm function. Marijuana lowers sperm count and motility through endocannabinoid system imbalance, while cocaine causes testicular damage and abnormal sperm morphology. Anabolic steroid use suppresses natural testosterone, leading to testicular atrophy, reduced sperm production, and possible long-term infertility.

2.2.3 Body Mass Index

The WHO classifies overweight and obesity as metabolic disorders because they involve abnormal or excessive fat storage and are influenced by a few environmental, hormonal, and

genetic variables. To assess if a person is overweight (BMI 25 kg/m^2) or obese (BMI 30 kg/m^2), body mass index (BMI) data is frequently employed. Although there is considerable debate on this subject, studies have revealed that obese men's sperm quality is worse than that of men who are of a normal weight [116].

Obesity is rising and connected to several health issues. BMI is a ratio of weight to height used to categorise people as overweight or obese. Due to its detrimental impact on male fertility, high BMI in men is associated with higher infertility rates. In evaluation to males of normal weight, overweight men had lower sperm concentrations, according to research by Jensen et al. on more than 1558 younger men [117]. Numerous more research have revealed a strong inverse association between BMI and sperm characteristics such azoospermia or oligozoospermia. But sperm morphology appears to be least impacted by high BMI. Even males who are underweight may have fewer effective sperm than others who are of normal weight. The study by Kort et al. likewise suggests a negative correlation between sperm motility and vitality and BMI [118].

Using two-dimensional difference gel electrophoresis (2D-DIGE), Kriegel et al. investigated the proteome alterations related to obesity in spermatozoa [119]. This study investigated the proteomes of two healthy donors with normal sperm and two obese individuals without diabetes whose sperm morphology was improving. Using matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS), nine different proteins that may be connected to obesity were found as part of the investigation. One of the proteins discovered in various gel sites during the study was outer dense fibre (ODF1), which is a structural protein found in the tail of sperm. These findings indicate that ODF1 may exist in diverse molecular forms due to proteolysis or post-translational modifications. Eight more proteins were identified, and analysis of their expression in obese patients showed that two were more expressed and six were less expressed. When compared to its expression in healthy donors, ODF1 expression in obese patients differed.

The team extended their research to a larger cohort by utilizing identical experimental techniques. They compared 21 healthy donors with normal sperm to 13 obese individuals without diabetes [120]. Seven proteins were found to have different expression levels between the two groups, apart from ß-Galactosidase-1-like protein (GLB1L), whose expression was decreased in obese patients. The presence of lactotransferrin (LTF) and semelogenin-1 (SEMG1), two of the six increased proteins, in many gel regions suggests that they were present

in a variety of molecular configurations. The sperm-specific glycolytic enzyme known to be crucial in sperm motility, spermatogenic glyceraldehyde 3-phosphate dehydrogenase (GAPDHS), was ten times more abundant in obese men.

The authors of the study discovered that clusterin (CLU), LTF, and SEMG1 were more prevalent and bound to the eppin protein complex (EPC) in obese people. The EPC protein is found in human seminal fluid and ejaculated spermatozoa. It is thought to preserve sperm and control motility. Modified versions of LTF and SEMG1 that were shown to be elevated in obese patients may have an impact on these people's sperm capabilities, according to Paasch et al. Pini et al.'s study used label-free quantification and LC-MS/MS analysis to investigate how obesity affects the sperm proteome [95, 121]. To reduce the impact of additional risk factors, the study only included men who were either obese or had a healthy weight and had normal sperm parameters. Patients with clinical problems such as diabetes and hypertension, as well as those who smoked tobacco, were excluded from the study.

The researchers analysed samples from five people in each group and found that, when compared to the control group, the spermatozoa from obese males contained 24 proteins that were less plentiful and three proteins that were more numerous [95]. The researchers used a fresh cohort of mass spectroscopy (MS) patients to carry out immunofluorescence and quantitative protein immunoassays on four particular proteins to validate their MS findings [95]. According to a literature review, the researchers proposed that stress and inflammation induced by obesity appears to change gene expression during spermatogenesis, which appears to cause modifications in sperm function. The review revealed that 14 of the proteins that showed differential expression were involved in various biological processes [95].

A different investigation examined changes in the proteome associated with asthenospermia induced by obesity (Table 2). Using label-free quantitative liquid chromatography tandem mass spectrometry (LC-MS/MS), the proteome of sperm cells of three significantly asthenospermic obese people has been compared to three clinically normal people [122]. Obesity-related asthenospermia was associated with 127 unregulated proteins, including 105 less abundant and 22 more abundant proteins than the control group. These proteins were found in proteomic studies of normospermia and asthenospermic samples [123].

Ferigolo et al. studied the seminal plasma of 20 healthy-weight and 27 obese men, excluding those with clinical disorders that would affect fertility [124]. One protein was unique to the

obese group, while 50 over-represented and 19 under-represented proteins were among the total 70 differentially expressed proteins. Functional enrichment study demonstrated that obese men's seminal plasma expressed more apoptotic pathway control, inflammatory and immunological reaction regulation, and antioxidant activity than control males.

In a mouse model, Peng et al. examined the sperm proteome's response to a high fat diet (HFD) [125]. The investigators used label-free LC-MS/MS to examine the proteome of the sperm cells from six mice given a conventional meal (CD group) and six fed a HFD group. The HFD group had 100 less proteins and 60 additional proteins, governing 160 proteins differently. The protein profile of spermatozoa from six mice fed a typical diet (CD group) and six mice fed a HFD were assessed using label-free LC-MS/MS. A gene ontology (GO) analysis found that most differentially expressed proteins were involved in intracellular protein traffic, shipping, cell structure and motility, endocytosis, as well as exocytosis. The researchers also investigated two cytoskeletal-related proteins, centrin 1 and CSP protein 1, which were found to be down-regulated specifically in the HFD group. The Western blotting confirmed this.

The researchers conducted a study on sperm from men categorized as normal weight and overweight or obese. They determined that the decrease in protein levels during spermatogenesis could account for the alterations in sperm morphology observed in men who are overweight or obese. In the meanwhile, Carvalho et al. used SDS-PAGE followed via LC-MS/MS analysis of protein bands to examine the proteome of spermatozoa from 10 rats in each group [126]. A comparison of protein abundance between the HFD and CD groups revealed a difference of 15 proteins, with seven proteins being less abundant and eight being more abundant in the HFD group. These proteins were largely involved in cellular respiration, metabolism, structural support, and defence. In summary Obesity, classified by WHO as a metabolic disorder, negatively affects male fertility by lowering sperm count, motility, and concentration. Studies show a strong inverse link between BMI and sperm quality, with obesity altering sperm proteins involved in metabolism, motility, and inflammation. Proteomic research in humans and animals reveals that obesity-induced stress and inflammation disrupt spermatogenesis, leading to structural and functional sperm defects that reduce fertility.

2.2.4 Diabetes as factor

Diabetes is a medical disorder characterized by insufficient insulin synthesis or inefficient insulin sensing, which controls blood glucose levels. Type-1 diabetes, which happens when the pancreas does not create enough insulin, and type-2 diabetes, which happens when the body

appears to not efficiently use the insulin that it produces, are two different types of the condition. Comparatively to men without diabetes, men with diabetes are more likely to experience infertility. According to numerous studies [38], diabetic males have lower sperm counts and motility, higher sperm death rates, and damage to their nuclear and mitochondrial DNA. Reproductive issues in diabetic men may have several contributing factors, including epigenetic dysregulation, diabetes, endocrine abnormalities, oxidative stress, and diabetic neuropathy.

Kriegel et al. conducted a study to investigate the effects of diabetes on the sperm proteome [119]. Using 2D-DIGE combined with MALDI-TOF-MS, the researchers analyzed the proteome profile of normomorphic spermatozoa collected from five normospermic donors and two individuals with type-1 diabetes in order to investigate any changes in the proteome during progression. In their study, the researchers recognized seven proteins that were differentially expressed, with two being less abundant and four being more abundant in diabetic men, while one protein was found to have varying relative abundance across three gel regions. A different research project undertaken by the same team involved examining the proteome of cleansed sperms from 36 individuals. This group involved 21 well healthy subjects, eight individuals with type-1 diabetes, and seven individuals with type-2 diabetes. This investigation was also mentioned previously in relation to the research on obesity. The root causes of reproductive problems in men with diabetes may be attributed to a diversity of aspects, including epigenetic dysregulation, diabetes, endocrine disorders, oxidative stress, and diabetic neuropathy.

Regarding type-1 diabetes, two proteins were found to be down-regulated, while two were up-regulated. Meanwhile, for type-2 diabetes, 12 proteins were up-regulated and 24 were down-regulated. Some proteins in both cases were discovered in different molecular forms, possibly due to post-translational changes or proteolytic breakdown. Similar to the obese group, diabetic men showed elevated levels of CLU, LTF, and SEMG1 and their modified forms, suggesting that the EPC complex might have been modified. Later, an et al.'s study compared the proteome of sperm from six men with type-2 diabetes and six healthy donors using isobaric tag for relative and absolute quantitation (iTRAQ) labelling and LC-MS/MS [127]. In diabetic patients, they identified 357 proteins with differential expression, where 319 proteins were up-regulated and 38 proteins were down-regulated. Four chosen proteins' differential expression was confirmed by Western blot studies [127].

To evaluate the influence of obesity and diabetes, Carvalho et al. investigated the proteome of sperm cells in rats [126]. Diabetes was induced through intraperitoneal injection of streptozotocin. Three of the 15 proteins that were deregulated in this investigation were more prevalent in diabetic rats compared to the control group, whereas 12 were less prevalent. In response to diabetes, they identified 82 dysregulated proteins, nine (11%) of which were common to both studies. The direction of variation in the abundance of some proteins differed between trials, like obesity research. Previous research had highlighted the impact of SEMG1 on sperm function, particularly in obesity. Although sperm viscosity has been linked to PIP, its precise function in sperm function is yet unknown [128].

Two separate studies that found opposing changes of keratin type II cytoskeletal 5 (KRT5) were independent of one another [127]. However, this protein is frequently found as a contamination in mass spectrometry tests, thus it should not be regarded as a sperm protein that is out of control. The cytoskeletal organization of the sperm tail is influenced by superficial solid fiber protein 2 (ODF2) and tubulin beta-4B chain (TUBB4B), which play significant roles [129]. As seen in the examined samples, the decline in these proteins in diabetes people may result in poor sperm morphology and motility. Serum amyloid P-component (APCS), on the other hand, was discovered to be more prevalent in the spermatozoa of diabetic men. This protein's density is positively correlated with sperm quantity, yet its significance asin seminal plasma is in doubt. Several metabolic processes, along with tissue homeostasis, apoptotic regulation, cytoskeleton, fertilization, and platelet degranulation, were enriched in humans as indicated by the GO analysis. Only the digestion of glucose and the hydrogen peroxide response were elevated in rodent dysregulated protein lists in one investigation [126]. These findings suggest that oxidative stress may be involved in the energy production disturbances seen in diabetic mice. Therefore Diabetes, caused by insufficient or ineffective insulin, negatively affects male fertility by reducing sperm count, motility, and DNA integrity. Both type-1 and type-2 diabetes disrupt spermatogenesis through oxidative stress, hormonal imbalance, and epigenetic changes. Proteomic studies reveal altered expression of key sperm proteins related to metabolism, structure, and inflammation, indicating that diabetes impairs sperm function and energy production, leading to infertility.

2.2.5 Use of mobile phone

Given that there are now much more people using mobile phones than there were only a few years ago, there are growing worries about the possible health dangers posed by their use.

Mobile phones use radiofrequency electromagnetic radiation (RF-EMR), which has risen in everyday radiation. A father's preconceptionally exposure to ionising radiation increases their likelihood of having a stillbirth, according to a study done on men who worked at the Sellafield nuclear station in Cumbria, UK [130]. Leydig cells, seminiferous tubules (ST), and sperm cells are especially vulnerable to cell phone damage in the male reproductive system. The usage of a mobile phone appears to cause scrotal heat and oxidative stress, which appears to lower testosterone biosynthesis, hinder spermatogenesis, and harm sperm DNA. In a study by, it was discovered that exposure to mobile phone radiation, DNA damage levels, and reduced sperm motility were all positively correlated [131].

Studies, however, have not discovered any appreciable effects of mobile phone use on sperm quality indicators. However, sperm quantity, semen amount, viscosity, liquefaction time, immotile sperms, and irregular morphology differed. It is thought that using a cell phone for extended periods of time or placing one in one's trouser pocket appears to cause hyperthermia and oxidative stress, which appears to have an impact on sperm production. Furthermore, radiation from mobile phones may injure cells by altering Leydig cells, causing seminiferous tubules to contract, or destroying DNA. Men who use their phones for over four hours a day had a 40% decline in sperm motility and viability [132]. Increasing mobile phone use raises concerns about male fertility due to radiofrequency electromagnetic radiation (RF-EMR). Prolonged exposure may cause heat and oxidative stress in the testes, leading to reduced sperm motility, DNA damage, and lower testosterone production. Men using phones for over four hours daily show up to a 40% decline in sperm quality.

2.2.6 Environmental factors

Humans receive exposure to exogenous and environmental chemicals differently. In both industrialised and developing countries, the chemical industry has grown quickly over the past 50 years, which has led to the release of numerous xenobiotics into the environment. These toxins damage the man reproductive system and appears to lead to infertility [133]. Pesticides, herbicides, preservatives, cosmetics, cleaning chemicals, municipal and private garbage, medications, and industrial byproducts are just a few of the foreign molecules that enter the male’s body systems in various ways. One potential factor in the rising prevalence of male infertility is exposure to chemical pollutants that mimic oestrogen and interfere with endocrine function. One significant factor is the impact of increasing temperatures on male factor infertility as human exposed different temperature variation in a day (see figure 14) [134].

Elevated scrotal temperatures, whether due to environmental heat exposure, appears to negatively affect sperm production and quality. Considering the significant sensitivity of mammalian spermatogenesis to testicular temperature, ambient temperature has been the primary meteorological variable in most research assessing the seasonality of seminal quality.

Humans may be vulnerable to trace levels of heavy metals from polluted food, water, air, and soil. Lead (Pb), cadmium (Cd), and mercury (Hg) harm male reproductive systems. Disrupting the hypothalamic-pituitary axis or directly affecting spermatogenesis causes low semen quality. Men's semen quality has been shown to decline after exposure to heavy metals [135]. Sallmen et al. found that lead exposure didn't just delay conception; it also induced infertility [136]. When the father welds stainless steel, there is a higher risk of spontaneous abortion; nevertheless, there is no increased risk in cases where the father welds other metals. There is widespread agreement that led exposure at high or even moderate levels appears to affect a person's ability to conceive.

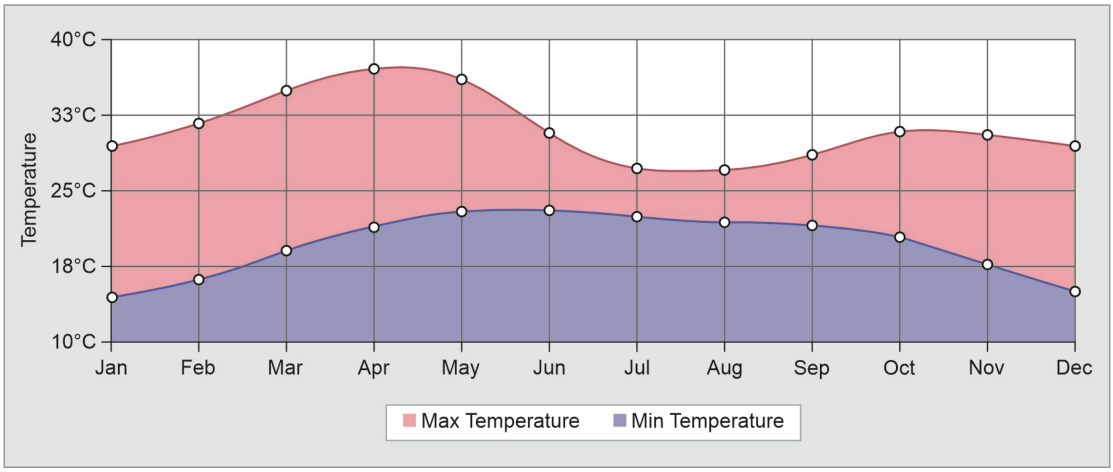


Figure 14 : Monthly average day and night temperatures

Lead levels in the blood that are higher than 40 g/dL have been associated to a decline in sperm count, according to research by Fatima et al. [137]. Entire blood specimens with lead contents above 35 g/dL had 50% reduced motility and 14% lower morphology. Telisman et al. discovered that having blood lead levels that high (36.7 g/dL) significantly decreased sperm motility and density [138]. Strong links exist between elevated lead levels and sperm destruction. The semen parameters appear to also be affected by high extents of cadmium. Men with high amounts of cadmium in seminal plasma (65 g/dL) had lower sperm counts and 36% more motile sperm, according to Akinloye et al. [139].

There is compelling evidence that sperm health appears to be negatively impacted by elevated mercury levels in the body. High blood-level mercury content (40.6 mmol/L) related to decreased sperm concentration, fewer than 50% progressive motility, and less than 14% normal morphology [140]. Lidia et al. found no correlation between metal contents and the total amount of blood, blood plasma, or seminal plasma [141]. The reproductive system is just one of the many body parts that are impacted by pesticide and other polychlorinated hydrocarbon exposure. Exposure to organophosphorus substances has an impact on spermatogenesis, testis weight, and sperm characteristics like density and motility, viability, and DNA damage. It may result in seminiferous tubule degeneration, modify testosterone, FSH, and LH levels, and reduce testes, epididymis, seminal vesicle, and ventral prostate volume. Pesticide exposure appears to also disrupt testicular antioxidant enzymes and prevent testicular steroidogenesis. These are a few potential ways that pesticides may impact the male reproductive system.

Compared to men in other occupations, agricultural workers have a risk of infertility that is more than ten times higher [142]. A father's preconception or earlier exposure to pesticides may also raise the likelihood of producing an anencephalic child. Congenital abnormalities are more likely to cause foetal death in men who engage in agricultural practises that involve the use of pesticides, especially when pesticides are employed often. It has also been noted that fathers seeking IVF therapy may have less fertile sperm due to exposure to pesticides. A higher incidence of acute lymphoblastic leukaemia has been linked to pre-conceptional paternal pesticide exposure in children under the age of 0.9 years. Tetrachlorodibenzo-p-dioxin (TCDD) is one of the most dangerous compounds produced by humans. A population exposed to TCDD was connected to a reduced male to female sex ratio in kids, with paternal exposure being more significant than maternal exposure, according to a study by Mocarelli et al. (2000) [143].

2.2.7 Medical history

Since conception needs the evaluation of both spouses and includes several organs, diagnosing male infertility appears to be difficult. A thorough medical history is the first stage in evaluating infertility, and it is divided into primary and secondary categories depending on whether the patient has previously experienced fertility. Both forms of infertility are assessed similarly, despite the fact that this classification appears to aid in reducing the differential diagnosis [144]. Testicular atrophy or poorer semen quality appears to result from childhood illnesses such cryptorchidism, post pubertal mumps orchitis, testicular torsion, or trauma. Male urogenital tract infections, such as prostatitis, urethritis, epididymitis, and orchitis, appears to

also cause infertility in men; in fact, one research of more than 4,000 infertile men found that male urogenital tract infections were as common as 35% [145].

The study of 1689 men discovered that 20% of primary infertile men had semen infections without symptoms [146]. Reduced sperm concentrations were associated with certain illnesses. Different sperm parameters might be negatively impacted by prostatitis, a common urogenital infection brought on by *E. coli*. Epididymitis is primarily brought on by *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in sexually involved male under the age of 35. In the meantime, *E. coli* is the most common infection discovered in infertile males over the age of 35.

When epididymitis or prostatitis is in its acute stage, semen analysis is not advised. Leukocytospermia, or an elevated white blood cell count ($>1x10^6$ per ml) in semen, is a term used to describe persons with chronic prostatitis or epididymitis. This indicates inflammation. This inflammation appears to be verified by a peroxidase test on semen. A thorough meta-analysis of 20 research including 5,865 males found that moderate to heavy smokers had lower-quality sperm [147]. Additionally, a negative connection between alcohol drinking and sperm parameters was discovered by another meta-analysis of 15 cross-sectional studies. Cannabis, the most commonly used recreational drug, appears to negatively impact male fertility by disrupting spermatogenesis, the HPG axis, and sperm functionality. Given the rising incidence of obesity in the world, researchers have also looked closely at the connection between obesity and male infertility.

Reduced sperm counts have been associated with obesity-related endocrine changes that cause peripheral testosterone to oestrogen conversion. A known risk factor for erectile dysfunction is metabolically unhealthy weight, which includes metabolic anomalies such diabetes, hypertension, dyslipidemia, and insulin resistance. Men having erectile dysfunction and metabolically healthy weight (no cardiovascular or metabolic disease) are at risk for metabolic issues. It is crucial to analyse the couple's sexual behaviours, including when coitus occurs and how well the erectile and ejaculatory systems are functioning. Ovulation tracking techniques should be used to make sure couples are effectively timing their sexual activity. To maximise the possibility of fertilisation, experts advise having sex every 48 hours around the period of ovulation [148].

Hypoactive sexual drive and a lack of sexual satisfaction, including pleasure, good sentiments, and orgasm, are the two sexual illnesses that affect men with infertility the most frequently.

Erectile dysfunction, early ejaculation, or both affect about one in six infertile men. Male infertility and its psychological effects appears to pose significant barriers to successful conception, hence they should be evaluated for during clinical examination [23]. Lots of peoples use advance sex comfort fancy items like lubricants, sex toys etc., but some are spermicidal. Instead of that couple appears to use natural fertility-friendly lubricants such Pre-Seed from ING Fertility in Spokane, Washington, are least spermicidal. Couples should use them cautiously and be warned [149].

2.2.8 Genetic factors

Infertility has been allied to several chromosomal abnormalities, together with deletions, inversions, mutations, aneuploidies, and translocations. According to one study, translocation occurs in infertile males at a frequency of 2.1% and is the most frequent chromosomal defect [150]. Chromosomal translocations cause 10% of male infertility, which appears to occur in a number of ways, including Robertsonian translocation or reciprocal translocation [151]. Robertsonian translocations, which involve acrocentric chromosomes, frequently cause oligospermia in carriers who otherwise have normal phenotypes. Infertility genetics are now recognised as a significant contributor to spermatogenesis disruption. Multiple levels of genetic damage in sperm appears to result in male infertility [152].

Disruptions in the genomic organisation inside sperm nuclei have been linked in several studies, both *in vivo* and *in vitro*, to adverse effects on sperm reproductive potential. The failure of *in vitro* fertilization (IVF) and intrauterine insemination (IUI) has also been linked to these illnesses. Additionally, the number of sperm in males seems to be inversely connected to hereditary variables. Previous studies revealed a bad association between sperm motility and DNA-damaged cells [120]. It is feared that male offspring who inherit these genetic defects may experience a more severe phenotype of infertility. Chromosome abnormalities affect between 2% and 8% of infertile guys, but have recently increased to 15% in azoospermia males [153].

For the management of male infertility, FSH monitoring is essential. FSH is crucial for starting the spermatogenesis process. Monitoring FSH levels in infertile men is essential for pharmacological and clinical management. Elevated FSH levels are linked to either normal or defective spermatogenesis, while normal FSH levels show damage of the germinal epithelium. Sertoli cell-only (SCO) syndrome or hypo spermatogenesis, which is characterised by a

reduced rate of spermatogenesis and may be accompanied by normal or increased FSH levels, may be visible on testicular histology [154].

ART with DNA-damaged sperm has a poor success rate for delivering healthy live babies. Recurrent ART failures appear to put a huge strain on a couple's finances, emotions, and mental health. Modern advances in molecular biology have revealed the hereditary reasons of male infertility. To detect genetic reasons of infertility, it is critical to assess infertile men comprehensively. Affected couples should be informed of the consequences of such diagnoses for the outcomes of ART and their future progeny. Male infertility has traditionally been linked to chromosomal abnormalities [155]. Karyotype abnormalities have been observed in 2% to 21% of infertile individuals, with a lower frequency in men with azoospermia. A comprehensive karyotype analysis of subfertile males was the first to link chromosomal irregularities to male infertility. Deletions in the long arm of the Y chromosome have been suggested as a potential cause of azoospermia. In mammals, a gene located on the Y chromosome plays a crucial role in testis development. Recently, three distinct loci related to spermatogenesis, known as azoospermia factors (AZF a, b, and c), were identified in separate regions along the Y chromosome (from proximal to distal Yq).

Immunogenetic disorders, chromosomal abnormalities, and significant genetic problems, including those that cause infertility, appears to develop from the buildup of heavy metals in the body and the oxidative stress that follows. Infertile men have a chromosomal abnormality rate that is 7% greater than the overall population [156]. More than 4% of male infertility is triggered by Klinefelter syndrome, which involves an extra X chromosome. In Klinefelter syndrome, two alleles of many genes linked to the X chromosome, which usually act based on disomy and are not inactivated through lyonization of the extra chromosome, may cause nuclear structure abnormalities that cause infertility. Only Turner syndrome-related mosaicism preserves partial fertility [157]. Men with azoospermia have 15% aberrant karyotypes and 5% oligozoospermia [158].

Male infertility appears to be caused by microdeletions on the Y chromosome or mutations in genes that govern male sexual development. These genes are located in the Yp11.2 region on the short arm of the Y chromosome, which also houses the amelogenin gene (AMELY locus) commonly used in forensic sex determination. In mammals, male sex is determined by the SRY gene on the Y chromosome, crucial for the proper function of the testes. The SRY protein, also called the testis-determining factor (TDF), is a DNA-binding protein that initiates male sex

determination. Additionally, cystic fibrosis is responsible for about 60% of obstructive azoospermia cases, further contributing to male infertility [159].

The genuine symptomatic form of cystic fibrosis, which causes infertility in 95% of affected men, is brought on by mutations in the CFTR gene. The reasons for male infertility are yet unknown, and environmental factors might have a big impact. The effects of element concentration, oxidative enzyme activity, and immunogenetic diseases on male fertility require further study. The development of new diagnostics and preventative measures depends on these studies in order to build research that appears to confirm the effects of environmental stressors on male fertility [160].

2.2.8.1 DNA methylation/demethylation

The latest results show that proper DNA methylation is necessary for conception and the growth of the embryo. In contrast, guys with idiopathic infertility and low semen quality had sperm with abnormal DNA methylation [161]. Numerous studies have demonstrated that fertile men's sperm have a stable single CpG methylation pattern, whereas sub fertile men show a dramatically changed pattern [162]. Notably, sperm motility and DNA integrity, as well as sperm chromatin and DNA methylation levels, are correlated [163].

Certain genes' promoter hypermethylation has been associated with low sperm quality and a higher risk of infertility. Houshdaran et al. (2017) found that sperm counts, motility, and normal morphology appears to be decreased by hypermethylation in the promoter regions of several genes [164]. The latest results show that proper DNA methylation is necessary for conception and the growth of the embryo. In contrast, guys having idiopathic infertility and low semen quality had sperm with abnormal DNA methylation in methylenate trahydrofolate reductase (MTHFR) gene. Which also causes idiopathic infertility [165]. These results show that the critical epigenetic aberration of MTHFR hypermethylation, which appears to lead to male infertility, must be addressed. According to a different recent study, mice with hypermethylated SOX30 genes have poor spermatogenesis, non-obstructive azoospermia, and their sperm are silenced [166].

2.2.8.2 Sperm DNA fragmentation

Despite the fact that SA is an essential part of the diagnosis of infertile men, its diagnostic efficacy is constrained due to inadequate discriminatory power and the inability to forecast the success of ART [167]. SA is essential for diagnosing male infertility, but it appears tonot

predict ART outcomes. SCSA, SCD, TUNEL, and COMET appears to be used to figure out fragmentation of sperm genome (SDF) instead of SA [100, 101]. Spermatozoa having DNA fragmentation become more prevalent in infertile men. High SDF is associated with low fertility rates after spontaneous or ART-assisted conception, intrauterine insemination, as well as miscarriage. ART patients should not undergo periodic integrity of DNA testing, considering that there is no clinically effective treatment for high SDF. Surgery to retrieve testicular sperm is risky and intrusive, hence there is little data on treatment outcomes, such as live birth rates. This method should only be utilized when ART has failed and all subsequent attempts to address the causes of damage to sperm DNA have failed [33]. In summary, Male infertility is often linked to genetic and chromosomal abnormalities such as deletions, translocations, and mutations that disrupt spermatogenesis and sperm DNA integrity. Karyotype anomalies, including Y-chromosome microdeletions and Klinefelter syndrome, are major causes of azoospermia and oligozoospermia. Epigenetic changes like abnormal DNA methylation (e.g., in *MTHFR* and *SOX30* genes) and sperm DNA fragmentation further impair fertility and ART success. Comprehensive genetic and molecular testing is crucial for accurate diagnosis, counseling, and management of affected men.

2.2.9 Air pollution

As it has a substantial impact on human health and has been connected to a few ailments, including respiratory issues, cardiovascular diseases, skin conditions, malignancies, and reproductive disorders, air pollution is an increasing concern in many places throughout the world. India in particular is the third most air-polluted nation, and it has grown to be a serious public health concern [168]. The causes of air pollution come from a variety of human activities, such as burning domestic waste, agricultural practises, waste treatment, and more. They also come from natural sources, like wind and volcanic eruptions. Air contaminants that harm human health include particulate matter, organic compounds that are volatile, ozone, the oxides of nitrogen, sulfur dioxide, carbon monoxide, polycyclic aromatic hydrocarbons, and an X-ray [169]. Male infertility appears to result from exposure to airborne particles because they appear to lower sperm motility, slow down normal sperm morphology, and increase sperm DFI.

In a current study that examined the connection amongst several gassy pollutants and semen quality, it was discovered that sulphur dioxide (SO_2) contact had a substantial detrimental effect on sperm quality over all exposure period. The study also found that sperm concentration and

motile sperm were significantly negatively impacted by both sulphur dioxide (SO_2) and nitrogen dioxide (NO_2), with the effects being more obvious through the early stages of spermatogenesis. These findings imply that gaseous contaminants significantly degrade the quality of semen, especially when sperm are developing [170]. In a different study, it was discovered that sperm kinetics, forward advancement, and total sperm motility were much lower in highway tollgate workers than in men from other areas. This was ascribed to the lead and nitrogen oxide emissions from automotive exhaust, which significantly decreased the general quality of the semen in these men when compared to controls [171]. Additionally, it was discovered that tollgate workers who were uncovered to car exhaust had higher levels of DNA fragmentation and disrupted sperm chromatin, indicating that this exposure may have a sizable genotoxic impact on human spermatozoa [172].

An increase in the proportion of guys seeking infertility treatment who have defective sperm morphology may be explained by ozone, a significant air pollutant that appears to lower the proportion of sperm per normal morphology. New research suggests that $PM_{2.5}$, a microscopic particulate matter that causes haze and air pollution, may also cause male infertility. $PM_{2.5}$ exposure increased sperm cells with cytoplasmic vesicles and the head morphological abnormalities. Several studies have found a negative relationship between $PM_{2.5}$ exposure and sperm motility, concentration, total sperm number, morphology, and semen quality [173-175].

Uncertainty surrounds the precise mechanism through which air pollution contribute to male infertility. Air pollution may modify sperm DNA, increase reactive oxygen species in the air, and disturb hormones, according to studies. Lead, zinc, copper, and polycyclic aromatic hydrocarbons (PAH) are examples of heavy metals found in air pollution that may have estrogenic, antiestrogenic, and antiandrogenic effects. These effects appears to cause aberrant gonadal steroidogenesis and gametogenesis, which appears to end in infertility [175]. Similar to this, $PM_{2.5}$ appears to build up in reproductive organs through the placental, blood-testis, or epithelial barriers and interfere with hormone levels, resulting in infertility [174]. In addition, oxidative stress brought on by air pollution appears to result in lipid peroxidation, sperm DNA breakage, and male infertility [176]. Male infertility is exacerbated by the creation of DNA adducts, which alter gene expression and DNA methylation [177]. Air pollution causes poor spermatogenesis, decreased sperm motility, abnormal sperm morphology, and increased sperm DNA fragmentation, which increases infertility among men.

Currently, a sizable portion of males struggle with idiopathic infertility. The most crucial clinical assessment of a man's ability to reproduce is still a routine semen examination, which counts the number of sperm, assesses their motility, and examines their morphology. The ions like sodium (Na), magnesium (Mg), potassium (K), calcium (Ca), iron (Fe) etc, are the most important among the sperm plasma's key components [178]. Each element has a distinct function in the body, and disrupting their balance appears to have negative effects. Ca, Mg, and other electrolytes participate in the transfer of nutrients and assist in preserving osmotic equilibrium. In redox processes, zinc (Zn) and Fe take part. Zn and Mg function as coenzymes for SOD, which helps shield sperm from the detrimental effects of free radicals by stabilising cellular membrane [168].

2.3 Evidence for impaired male reproductive health over year by year

According to Carlsen and his team, male fertility had significantly decreased by 1992 [179]. Their research revealed that the sperm concentration of healthy men decreased from 113 million/ml in 1940 to 66 million/ml by 1990. Sperm quantity decreased worldwide, especially in Ibadan, Nigeria (71.2 million/ml), Lagos, Nigeria (54.6 million/ml) [180], Salem, Libya (65.0 million/ml), Dar es Salaam, Tanzania (66.9 million/ml), and Copenhagen, Denmark. Semen samples should have above 40 million sperm per milliliter under ideal conditions. According to WHO guidelines, a normal semen sample must have 20 million sperm per ejaculate, 50% motility, and 60% normal morphology. Less than 20 million sperm per milliliter of discharge may affect fertility, although 5 to 10 million appears to cause pregnancy if the sperm have forward motility. Some andrologists say 10-15 million sperm per milliliter is adequate.

On the other hand, some claim that 48 million and 55 million sperm per millilitre are the lowest numbers for a normal sperm count they have changing sperm proteins also [33]. However, only a small percentage of men in today's Western industrialised nations have sperm values that meet these optimum levels, according to the literature that is currently accessible on sperm count. Other sperm metrics, like sperm motility and typical sperm morphology, are also declining; the declines are not just in sperm count. According to a Danish study, between 1966 and 1986, the percentage of defective sperm rose from 40% to 59%. Furthermore, a 1988–2007 study at the Reproduction Biology Laboratory of the University Hospital of Marseille in France

found reducing patterns of total sperm count (1.6% / year), total motility (0.4% / year), rapid motility (5.5% / year), and normal shape (2.2% / year) in the overall population. [181].

According to research done in India, between 30% and 40% of males in the reproductive age range have a considerable drop in sperm quantity and quality [182]. A prior study in Jaipur, Rajasthan, indicated that male infertile (35% of them) had the highest rate of azoospermia [120]. As in other regions of the world, urban areas like Mumbai, Bangalore, and Jalandhar have been observed to have similar incidence rates of azoospermia and oligozoospermia. Kurnool and Jodhpur, respectively, had azoospermia prevalence rates of 38.2% and 37.3%, according to Mehta et al. [183]. Though greater than elsewhere, azoospermia occurrence in Jaipur is still within the range found in Indian data [184].

2.4 Global approach toward deregulated protein cause male infertility

Males are deemed infertile if their sperm parameters are below the WHO normal threshold [185]. These criteria, which are the most important ones, include defective sperm morphology (teratospermia), low sperm concentration (oligospermia), and poor sperm motility (asthenospermia). Semen quantity along with seminal markers of epididymal, prostatic, and seminal vesicle activity are less significantly connected to infertility. Low sperm concentration, which causes 90% of male infertility issues, is the main factor, and aberrant semen characteristics are positively connected with sperm count. Underlying causes of these issues include disruption of pre-testicular, testicular, and post-testicular components that influence sperm count, motility, and morphology.

Semen assessment, that has a sensitivity level of 89.6% and appears to recognize 9 out of 10 male infertility cases, remains the most essential and valuable investigation. However, the results of this test do not indicate fertility [186]. It solely offers details on the makeup and development of sperm as well as how they interact with the seminal fluid. While it gives information on sperm production (count) and quality (motility, morphology), it does not evaluate the spermatozoa's functional capacity to proceed through additional maturation procedures required for fertilisation. The WHO revised minimum reference limits and 95% confidence intervals (Cis) from studies involving over 1900 males who had partners who had an average time-to-pregnancy of less than 12 months for semen analysis.

In aseptic circumstances, the subjects submitted semen samples by masturbating into a sterile collection semen container. They had forty-eight hours to refrain from having sex before collecting the specimens. The samples were then given at least 20 minutes, but no more than an hour, to liquefy in a 37°C heating stage of laminar air flow before undergoing a standard semen analysis, which included determining the colour, volume, pH, appearance, consistency, agglutination, sperm count, motility, vitality, and morphology of the semen [187].

According to a study, 48.5 million couples that participate in unprotected sexual activity experience infertility worldwide [69, 188]. However, this statistic does not break down infertility rates by topographical location. Additionally, many clinical research adheres to the WHO definition of infertility, which states that it occurs when a woman is unable to conceive after a year of trying. However, demographic studies take a five-year look at infertility rates. Sharlip's study, indicates that approximately 50% of infertility cases are caused solely by female factors, 20% – 30% by male factors, and the remaining 20% – 30% by a combination of both male and female factors [69]. As the 'Sharlip factor' is commonly cited and widely recognized in discussions of male infertility, it served as the basis for our calculations [69].

Table 2: Worldwide proteomics to discover altered sperm proteins and male infertility associated risks

Authors	**Organism**	**Processing of Sperms**	**Outcomes**	**Proteomics Approach**
			Obesity	
Ferigolo et al., 2019 [101]	Human	Centrifugation	The research discovered 485 proteins, 70 of which revealed differential expression. These included 50 more abundant proteins, 19 less abundant proteins, and 1 protein discovered only in fat people.	LC-MS/MS (hybrid quadrupole-Orbitrap) was employed in combination with in-solution digestion for the analysis.
Kriegel et al., 2009 [95]	Human	50–90% gradient	In obese males, 2700 fluorescent protein notices were found, with 9	DIGE and MALDI-TOF-MS used to

			differently expressed proteins, 2 more common and 6 less abundant, and one gel spot-dependent.	analyse 2D-PAGE results
Liu et al., 2015 [99]	Human	45% Percoll gradient	1975 proteins identified, with 127 being differentially expressed: 22 more number and 105 less number in obesity-associated asthenozoospermia.	LC-MS/MS (LTQ-Orbitrap) after FASP
			Diabetes	
Kriegel et al., 2009 [95]	Human	50–90% gradient	2700 fluorescent protein spots discovered, 7 proteins differently expressed (4 greater in number, 2 fewer in number, and 1 based on gel spot) in diabetic men.	DIGE 2D-PAGE; MALDI-TOF-MS
Paasch et al., 2011 [97]	Human	50–90% gradient	3187 fluorescent protein spots detected in diabetic patients. Type-1 diabetic patients had 8 differentially expressed proteins: 6 more abundant, and 2 less abundant. Type-2 diabetic patients had 39 differentially expressed proteins.	Using 2D-PAGE (DIGE) and MALDI-TOF-MS, the proteins were analysed.
An et al., 2018 [105]	Human	Centrifugation	Differentially expressed: 319 more abundant, 38 less abundant proteins	LC-MS/MS (hybrid quadrupole-Orbitrap) with gel-free digestion

			were identified from 1114 proteins in diabetic men.	
Tobacco Smoking				
Antoniassi et al., 2016 [110]	Human	Centrifugation	Proteins identified: 422. Differentially expressed proteins: 25. Absent: 1. More abundant: 6. Less abundant: 18. Method: Gel-free digestion; LC-MS/MS.	LC-MS/MS (hybrid quadrupole-Orbitrap) with in-solution digestion.
Chen et al., 2015 [111]	Mouse	Swim up	1000+ protein spots, 22 differently expressed proteins: ten more abundant, Twelve fewer in treated mice.	MALDI-TOF-MS and 2D-PAGE
Bisphenol-A exposure				
Rahman et al., 2016 [187]	Mouse	Swim up	399 spots detected, with 23 differentially expressed proteins in spermatozoa exposed to the upper concentration of BPA: 8 more abundant and 15 less abundant.	ESI-QTOF, 2D-PAGE
Rahman et al., 2017 [188]	Mouse	Swim up	6 proteins differentially expressed in males from BPA-exposed females, detected from 284 spots by 2D-PAGE	2D-PAGE and ESI-QTOF

Currently, there is no specific information on the occurrence of male infertility universal. As a result, employed the same estimation parameters as earlier research, specifically the "Sharlip factor," to determine the data in this paper [189]. They approximated male infertility rates based on the assumption that 20–30% of infertility cases were caused by male causes in locations

where male infertility rates were not available. In those places, authors utilised the published female infertility rates. In Sub-Saharan Africa, for example, 14.2% of women reported infertility. If 50% of infertility cases were caused by a combination of male and female factors, we calculated that 2.1% and 4.3%, respectively, of the region's total infertile population were due solely to male factor infertility and any form of male factor involvement.

2.5 Techniques used in assessing the semen quality

2.5.1 Assessment of functional characteristics

The assessment of the functional parameters evaluated through makler chamber and Diff Quick staining method, which are described below.

2.5.1.1 Evaluation of sperm count and sperm motility by using makler chamber

Makler chamber is used for the sperm count and motility by using raw semen samples. It having depth of 10 ul, sample of same size appears to be used for analysis of same parameters without dilution as shown in figure 15[190].

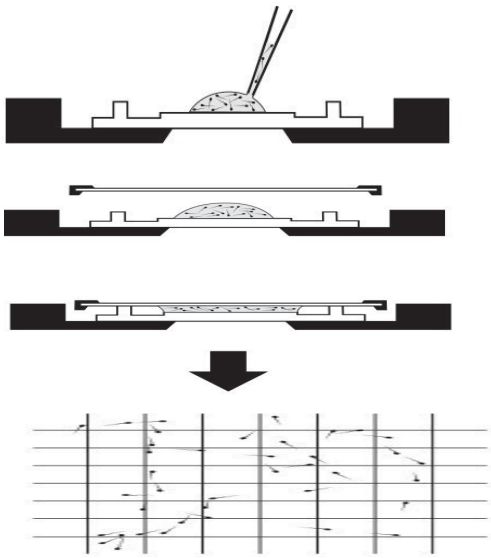


Figure 15: Makler counting chamber cross-section and microscopic diagram

2.5.1.2 Morphological assessment by using Diff Quik staining methods

Morphology of spermatozoa evaluated by using diff quick staining method in which abnormalities of head, midpiece and tail assed. As shown in figure 16, Head abnormalities could be large, small, pinhead and double head which shown by red colored arrow. Midpiece abnormalities may be small and expanded was shown by yellow color and blue color used to show abnormality into tail regions[191].

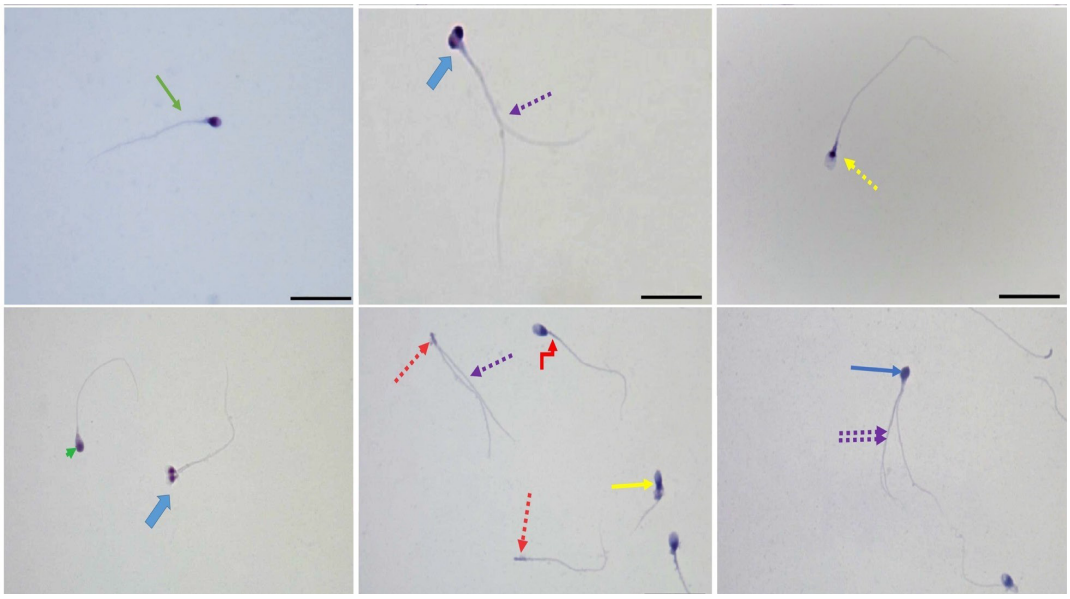


Figure 16: Representation of microscopic morphological defects in spermatozoa with different colour

2.5.2 Evaluation of the Sperm DNA Damage

The highly specialised spermatozoon cell transports genetic information to the haploid oocyte. Unlike somatic cells, spermatozoa have a tighter and more condensed chromatin structure, resulting in a smaller nucleus and less vulnerability to ionising radiation. The sperm DNA's integrity must be preserved until it is delivered to the egg. After fusing with the egg membrane, the sperm chromatin must speedily decondense to release the sperm DNA. However, the proper condensation and recondensation of sperm chromatin remains vital for sperm function. When analysing sperm DNA, if the sperm chromatin is degraded or damaged, the results may be erroneous [192]. DNA damage may cause due to following mechanism.

a) Single strand or double strand breaks DNA of spermatozoa

b) ROS causing DNA damage

c) apoptosis during spermatogenesis causes fragmentation in DNA

d) Defective maturation causing DNA impairment

e) Environmental contaminants damages to DNA

Single strand (ss) or double strand (ds) breaks in DNA, base removal or alteration, besides DNA cross-linkage with inter- or intra-strand, appears to all result from chromatin damage [193]. Sperm DNA fragmentation (SDF) is closely linked to ss or ds DNA breaks [194].

While single-strand fractures are thought to be less hazardous, double-strand breaks appears to disrupt implantation rates, embryo development, and are linked to recurrent miscarriages. High numbers of single-strand breaks appear to have a deleterious impact on normal pregnancy outcomes as well as sperm motility progression [195]. However, most methods for assessing SDF are unable of distinguishing between break in single-strand as well as double-strand. SDF may be further made during spermatogenesis via abortive apoptosis that makes sure that defective germ cells not develop into spermatozoa; nevertheless, the failing of process accumulates spermatozoa displaying apoptotic markers in the ejaculated semen figure 17 [196].

SDF in sperm appears to be triggered by intrinsic factors like faulty maturation of germ cell, apoptosis during spermatogenesis, and OS, as well as exogenous causes such as smoking, heat exposure, environmental contaminants, and chemotherapeutics [197]. Male infertility shows OS as a prominent contributor, with excess ROS generating a variety of negative consequences, including SDF [198]. Mismatches, loss, and modifications in base, with DNA crosslinks and adducts, pyrimidine dimers, single and double strand breaks (SSBs and DSBs), may all cause SDF as well as potentially compromise natural conception or ART outcomes as shown in figure 18 and 19 with Single and double strand DNA fragmentation. Abortive apoptosis during spermatogenesis appears to also cause SDF since it ensures no development of defective germ cells into spermatozoa. It might accumulate spermatozoa articulating apoptotic indicators in the ejaculated semen after failing of process [197].

Increased SDF levels have been related to male infertility then a variety of illnesses, including varicocele, infection in accessory gland, cancer, progressive paternal age, long-lasting sickness, acquaintance to environmental chemicals, as well as standard of living factors [19]. Increased SDF levels have also been proven to have an adverse effect on conception rates as well as offspring health and well-being [199, 200]. As a result of the unfavourable influence of SDF on fertility in male, more clinicians may use SDF testing in clinical settings [201]. There has been a prominent upsurge in the numeral of research demonstrating a link between SDF and male infertility during the last 20 years. During this time, the primary focus of SDF research has been on lifestyle variables, varicocele, and asthenozoospermia [197]. Interventions to increase reproductive results and promote healthy offspring have also been investigated. SDF is induced by two factors including improper maturation and abortive apoptosis[202]. Defective maturation occurs within the testis and is characterised by chromatin compaction difficulties, which are often resolved as a result of histone interaction involving transitional proteins as well as protamine's [203].

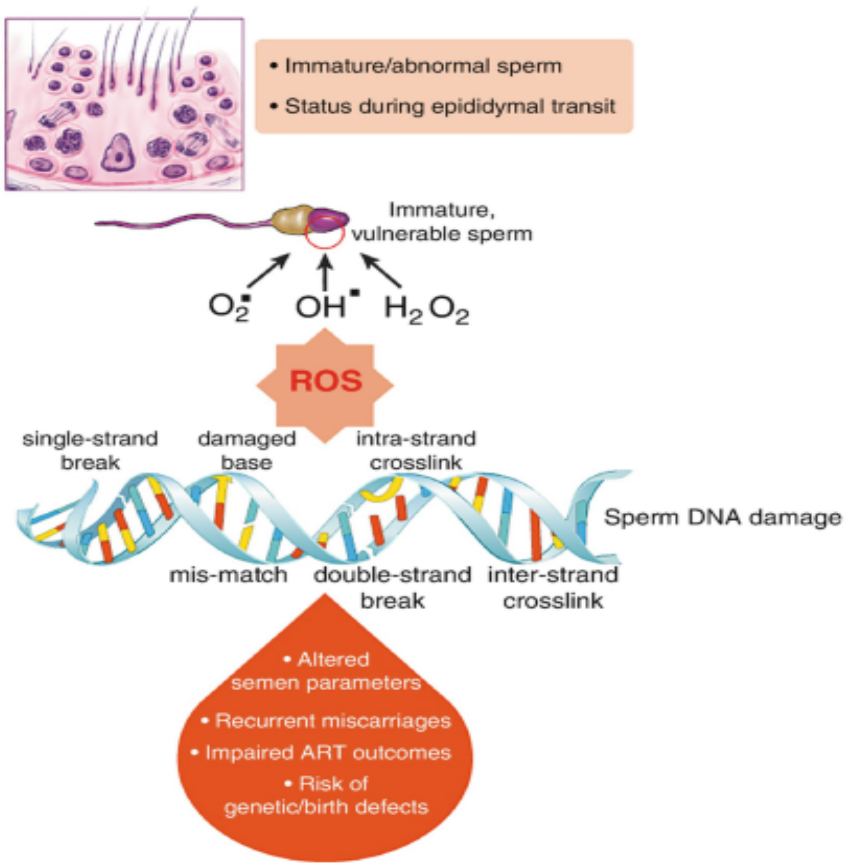


Figure 17: Sperm DNA fragmentation causing male infertility

The action of the endogenous nuclease topoisomerase II, generating DNA breaks to relieve torsional stress that allow proper histone disintegration with chromatin packaging, is required for this process [204]. If these fractures are not healed, they appears to result in reduced chromatin packaging, improper maturation, and a higher presence of sperm containing SDF in the ejaculate [205]. It appears to potentially be persuaded by apoptosis in spermatogenesis. Apoptosis is the process by which faulty germ cells are eliminated, preventing them from maturing into mature spermatozoa. However, if this process fails, apoptotic spermatozoa appears to collect in the ejaculated semen [206]. Extrinsic apoptosis takes place when Fas-ligand interacts to death receptors [207]. Fas expression in ejaculated sperm implies an increase in abortive apoptosis. Excess ROS appears to harm DNA then trigger apoptotic pathways in spermatozoa [208, 209]. Furthermore, oxidative stress contributes to SDF by inducing the formation of DNA adducts, leading to DNA damage. One example of such adducts is 8-hydroxy-2-deoxyguanosine (8OHdG), which forms due to direct oxidative damage to DNA bases, especially in regions where protamine protection is insufficient [210].

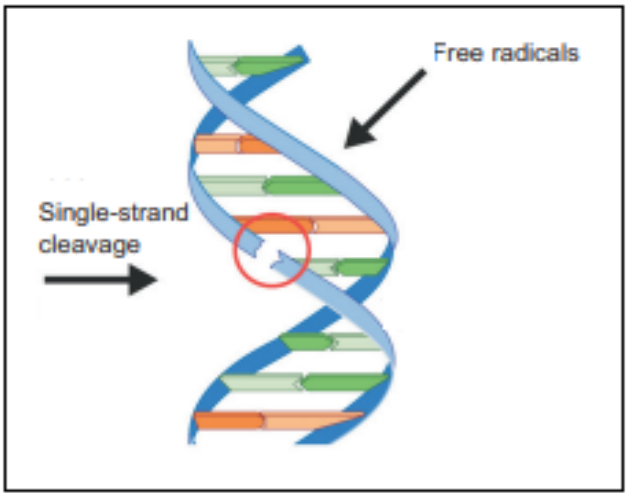


Figure 18: Single strand DNA fragmentation [193]

OS additionally stimulates the mitogen activated protein kinase (MAPK) pathway, inducing apoptosis and hindering maturation [211]. In spermatozoa, OS triggers intrinsic apoptotic pathways, with phosphatidylserine externalisation serving as an initial indicator and SDF serving as a late hallmark of apoptosis [212]. The process starts through a mitochondrial pathway where cytochrome c is released into the cytosol, leading to the activation of caspases 3, 6, and 7 through proteolytic cleavage [213].

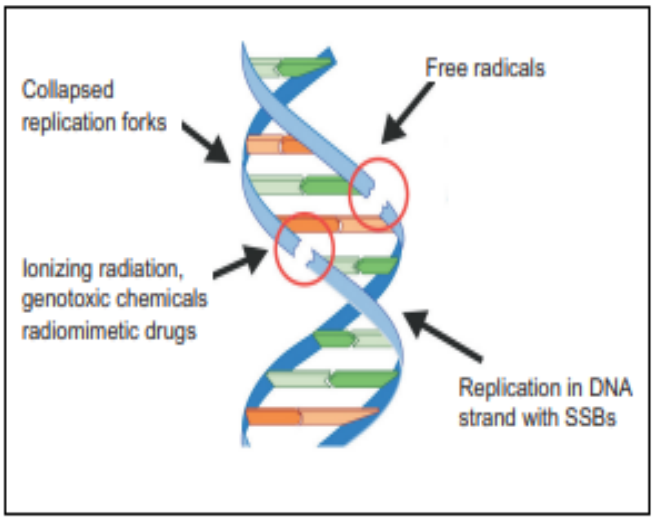


Figure 19: Double strand DNA fragmentation

Environmental contaminants, caspase with endogenous endonucleases, mistakes of replication, UV rays, as well as ionising radiation are all potential reasons of sperm DNA damage as shown in figure 20 [214]. DNA fragmentation of single-strand and double-strand DNA (ssDNA and dsDNA) is both damaging and unstable. However, dsDNA fragmentation is a permanent form

of damage with a negative impact on fertilisation affecting embryo growth. As shown in the figure 18 and 19, critical DNA repair mechanisms including ssDNA and dsDNA break repair, nucleotide excision repair, and mismatch repair are used in counteracting the DNA damage process and maintaining DNA integrity [193, 202]. Impaired DNA repair systems result in the production of defective spermatozoa with significant percentage of DNA damage [215].

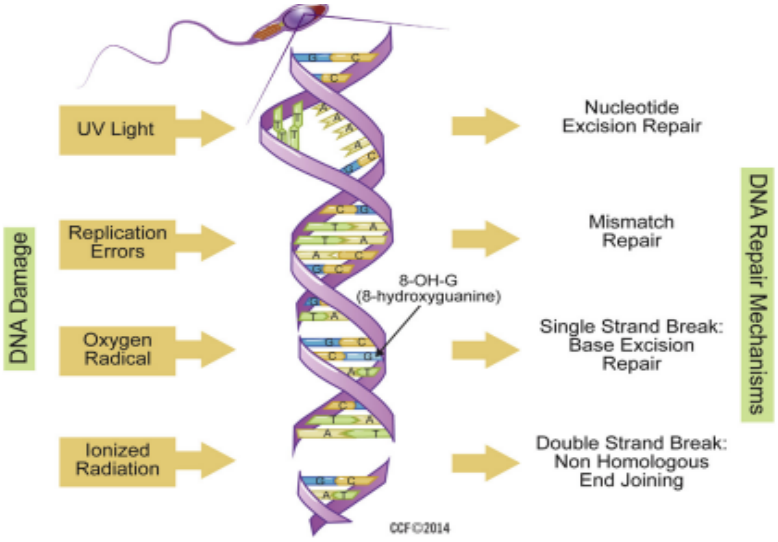


Figure 20: DNA damage and repair mechanisms

SDF testing is a vital sperm function assessment that supplements traditional sperm analysis. Elevated SDF levels have been linked to poor sperm quality, poor fertilisation, deprived embryo quality, and poor pregnancy results. Various SDF assays have been created and intensively researched over the years for pertinence and correctness as innovative assessments of sperm function. Various procedures for assessing SDF percentage have been established, and they differ from each other.

- Sperm chromatin structure assay (SCSA).
- Comet assays.
- Transferase UTP nick-end labelling (TUNEL).
- Sperm chromatin dispersion (SCD).

2.5.2.1 Sperm chromatin structure assay

SCSA is a well-researched examination that has been used to spot sperm DNA loss for over 30 years. It is an indirect assay in which the DNA is exposed by denaturation via heat or acidic treatment, allowing for the detection of breaks in ssDNA. When a fluorescent stain called AO

(acridine orange) fixes to ssDNA, or dsDNA, it emits green and red fluorescence respectively. The flow cytometry is then used to examine the stained sperm cells [216].

The SCSA sets a clinical criterion known as the DFI or DFI at 30% recognized on the percentage of sperm with red staining suggesting damage in DNA. SCSA is applicable to both fresh and frozen materials. A strong correlation (R^2 = 0.98) amongst authorised laboratories indicates that it is a reasonably straightforward test with great repeatability and reproducibility [217, 218]. Furthermore, the SCSA has higher precision, with a coefficient of variation of about ~1-3%.

In contrast to the TUNEL assay, which produces DFI values ranging from 4% to 36%, the SCSA has established DFI determination threshold ranges of 20-30%. Fernandez et al.'s SCD assay is a secondary technique for calculating SDF [219, 220]. Loading intact DNA into agarose and denaturing it with an acidic solution results in halos or chromatin dispersion caused by relaxed DNA. Figure 21 Fluorescence microscopy is used to visualise this dispersion [221]. Spermatozoa with fragmented DNA do not display this dispersion and instead have non-dispersed chromatin, which is indicated by tiny halos. The quantity of non-dispersed chromatin in sperm is proportionate to the extent of ssDNA damage.

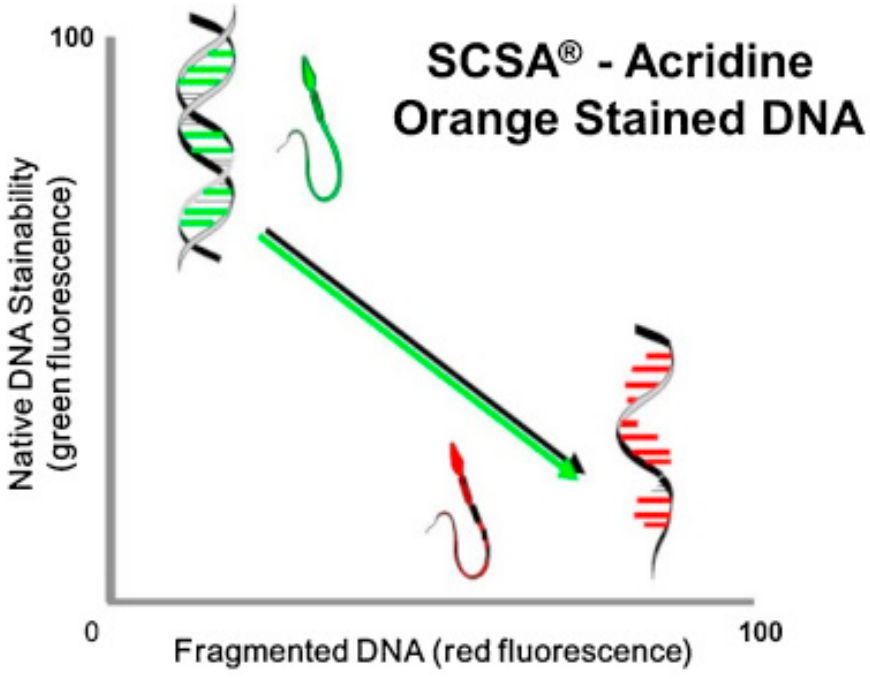


Figure 21: Fluorescence colour indicating normal DNA as green to fragmented DNA as red

The SCD assay may be done on unwashed as well as washed sperm samples. The concentration of sperm is initially increased to 10^6/ml. 20 µL. At 37 °C, a 20 L of diluted spermatozoa sample is combined with 80 L low melting agarose of 1%. This mixture is spread over 50 µL of pre-coated agarose slides and permitted to harden for four minutes at 4 °C before being enclosed by a coverslip. Denaturing the DNA at the next step includes submerging the sperm-entrenched agarose in an acidic solution of 0.08 M HCl for seven minutes in an obscure environment at 22°C. This is treated for 15-minutes at room temperature using neutralising as well as lysing solutions to stop the denaturation process. The samples are then rinsed in a Tri s-borate-ethylenediamine tetra acetic acid (EDTA) buffer for 2 minutes before being rehydrated with ethanol in increasing concentrations (70, 90, and 100 percent). Lastly, DAPI or 4', 6-diamidino-2-phenylindole, is applied to stain the spermatozoa and examined below a fluorescence microscope [222, 223].

2.5.2.2 Comet assay

The Comet assay, frequently referred to single-cell gel electrophoresis, entails embedding sperm in an agarose gel, then performing detergent lysis. In this experiment, fragmented DNA migrates as a tail, whereas entire DNA persists in the head. Ostling and Johanson were the first to propose it in 1984 [224]. Smaller DNA strands move further from the head during electrophoresis than larger DNA strands. The degree of SDF within each spermatozoon is directly connected to the tail length and fluorescent staining intensity. This technique may identify different kinds of fragmentation in DNA and is applicable to fresh sperm samples. It needs at least 5000 spermatozoa, making it possible to use oligozoospermic samples [225].

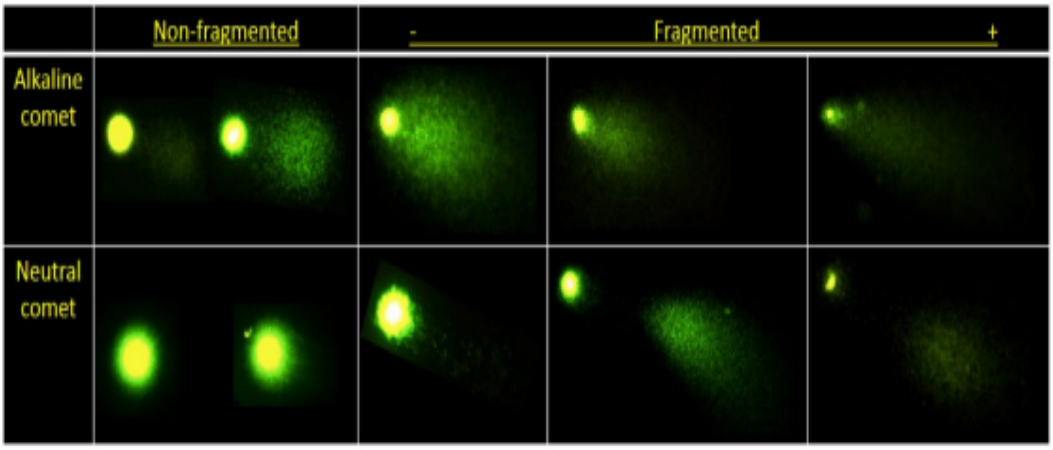


Figure 22: Summary of the various comet-shaped sperm cells in the neutral and alkaline comet assays, ranked in order of increasing DNA fragmentation

The Comet test is a flexible technology allowing for differentiation between single-strand and double-strand breaks. It entails embedding spermatozoa in an agarose gel, then nuclear decompaction and electrophoresis under alkaline conditions to detect SSBs or neutral conditions to detect DSBs (see figure 22) [226] [227]. DNA fragments (comet tail) migrate away from intact chromatin (comet head), resulting in a comet-like structure that allows cells to be differentiated as positive for fragmented or negative for non-fragmented [228]. Fluorescence microscopy is used to stain and see individual comet-shaped spermatozoa, and the percentage of shattered cells is calculated.

Moreover, protamine extraction and quantification were carried out according to established techniques. The study team used the protamine extraction techniques developed by Soler-Ventura et al. [229]. Protamine extraction was performed in a subgroup of samples from each study group including high and low DSBs and SSBs, high DSBs and high SSBs, with at least 3 million spermatozoa available following Comet sperm DNA fragmentation evaluation. Acid-urea polyacrylamide gel electrophoresis is used to quantify the removed protamine together with cumulative amounts of a standard human protamine. The protamine-extracted DNA pellet is used for DNA quantification using 0.5 M perchloric acid hydrolysis for 20 minutes at 90 °C. A NanoDrop spectrophotometer is used to detect absorbance at 260 nm to normalise the quantity of protamines with the quantity of DNA in each sample.

Figure 23 shows how perform the Comet assay; individual spermatozoa are suspended in agarose with low-melting at 37°C. The blend is then located on a microscope slide coating with a coverslip of glass. After the slides are refrigerated to 4 °C to allow the agarose to solidify, the spermatozoa are lysed in a solution comprising Triton X-100 detergent with proteinase K [230]. For 20 minutes at 25 V, the micro-slides are electrophoresed in a neutral buffer, causing fragmented DNA for migrating to the anode pole. The slides are first soaked in a denaturing solution comprising 1 M NaCl and 0.03 M NaOH for 2 minutes and 30 seconds at 4 °C, followed by 4 minutes of electrophoresis in a 0.03 M NaOH buffer at 20 V in the alkaline Comet assay [231]. SYBR Green, I dye the slides after electrophoresis and a fluorescence microscope is used for viewing the fragmented DNA. The data analysis uses the length of the tail, which appears to be done manually or by specific marketable accessible software [222].

A data analysis includes a histogram graph generated to show the frequency distribution of the percentage of cells in the population that had single-strand (SSBs) breaks or double-strand (DSBs) breaks. The population is categorised as high, medium, and low, based on the fraction

of fragmented cells. The terciles approach [227], identifies the thresholds. Samples chosen and stratified based on high levels of DSBs, SSBs, or both, resulting in four categories as high and low DSBs and SSBs, high DSBs, and high SSBs. Several indices, including SDF, as well as concentration, motility, vitality, and morphology of sperm, with sORP/c (sperm concentration), P1/P2 ratio that is linked with high SDF levels, transformed seminal parameters, in addition low ART success rate [193, 232], and protamine (P1 -mature sperm protein, P2- immature sperm protein, and P1 + P2) to DNA content of the native semen, were investigated.

There is no significant alteration observed between the state of protamination and the kind of DNA fragmentation. This illustrates that increased DNA damage and different types of breaks may not always be associated with changes in the P1, P2, P1+P2, or P1/P2 ratios. Different types of protamines and post-translational modifications, such as phosphorylated protamines, may impact the complexity of sperm chromatin structure. To completely understand the mechanisms driving sperm chromatin structural anomalies and their influence on male infertility, more research is required.

2.5.2.3 Transferase UTP nick-end labelling assay

The TUNEL assay is a dependable approach with good accuracy that appears to be used as a secondary diagnostic test in Andrology laboratories. It has the capacity to predict fertility results when combined with basic sperm analysis, enabling the selection of appropriate ART procedures for infertile couples. Furthermore, TUNEL assay has the potential as a prognostic test, providing crucial information for personalised treatment decisions in men with infertility.

The TUNEL assay has increased therapeutic utility for determining SDF in infertile men. It uses a microscope or a flow cytometer, and precisely targets DNA strand breaks in sperm DNA. Although the microscopic assay appears to be done on clean, washed samples, or cryopreserved samples, the more accurate and sensitive assay is based on flow cytometry (see figure 23) [222]. The TUNEL assay has been standardised at the American Centre for Reproductive Medicine, Cleveland Clinic, using a benchtop flow cytometer with established reference values [233].

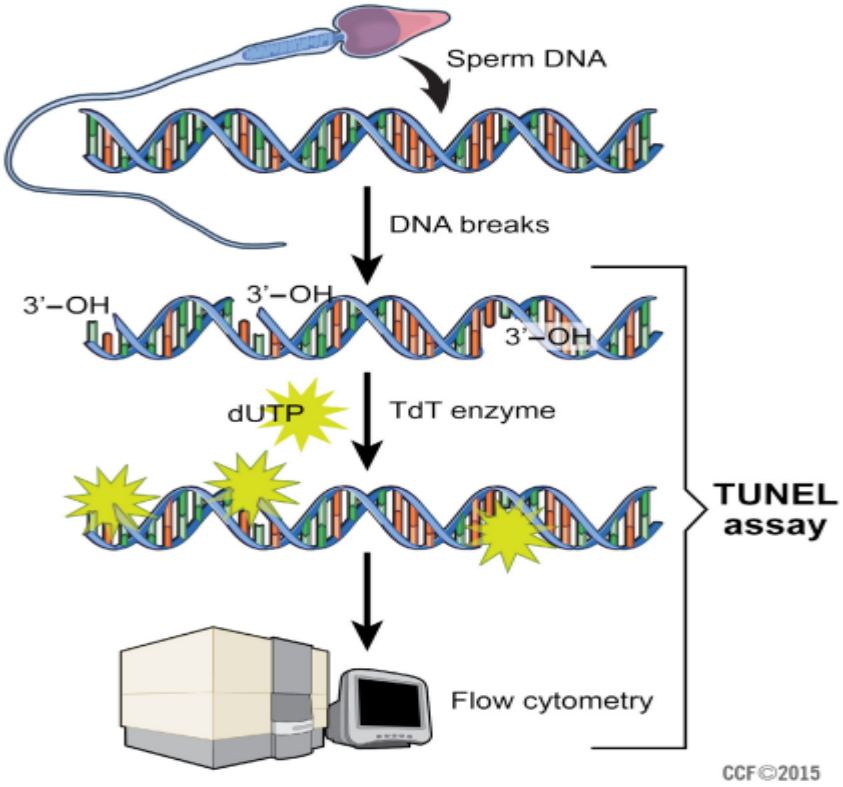


Figure 23: Sperm DNA Staining with flow cytometer for analysis

A template-independent DNA polymerase, TdT or terminal deoxynucleotidyl transferase, is added to the 3' hydroxyl (OH) breaks-ends of single and double stranded DNA in the TUNEL assay [234]. Fluorescein isothiocyanate (FITC) is coupled with dUTPs, and the fluorescence signal detected through flow cytometer correlates to the SDF in the investigated spermatozoa [215]. For nucleic acid staining, a red-fluorescent dye, a counter stain, and PI (propidium iodide) is utilised.

SDF with TUNEL was recently standardised utilising a tabletop flow cytometer and kit of Apo-Direct. A least of 2×10^6 sperm/ml from the liquefied sperm sample is aliquoted and fixed in 1 ml of 3.7% paraformaldehyde to perform the experiment. Then for separating spermatozoa centrifugation is applied for 4 minutes at 600g before being incubated for 30 minutes at -20°C using ice-cold 70% ethanol. Subsequent incubation, the samples are centrifuged at 300g for 7 minutes to eradicate the supernatant while preserving the pellet of sperm. To remove the wash buffer, the pellet is resuspended in 1 ml of wash buffer and vortexed and centrifuged as before. The pellet is resuspended in 50 µL of staining solution. The positive as well as negative control cells included with the kit (6553LZ and 6552LZ) are also stained with FITC-dUTP. Unreacted

FITC-dUTP is removed after a 60-minute incubation at 37 °C using centrifugation with 300g for 7 minutes and rinse it using 1 ml of rinse buffer. Lastly, the resuspended cell pellet in 0.5 ml of PI/RNase staining buffer is incubated for 30 minutes at normal temperature.

The SDF is analysed using a flow cytometer (BD Accuri C6) that examines cells for physical as well as fluorescent characteristics. The fluorescence signals created by the tagged cells are used to sort spermatozoa in the flow channel. Each sample, as well as both +ve and -ve controls, is run twice. The 488 nm FL1 laser detector include standard band pass of 533/30 nm to catch FITC signals with green fluorescence, while FL2 detects PI produced red fluorescence using a conventional 675/25 nm band pass. At least of 10,000 incidents are investigated, and positive tested spermatozoa for TUNEL are thought to exhibit DNA fragmentation. The SDF percentage in discrete sperm samples is determined using the user-friendly Zoom tool programme that is pre-installed [233].

2.5.2.4 Sperm Chromatin Dispersion method

In clinical practise, various SDF techniques are used. TUNEL is performed by labelling free 3'-OH nicks with dUTP [235]. SDF is distinguished by the Comet assay using DNA electrophoretic separation, where a comet-like profile is exhibited by damaged DNA. Following the elimination of DNA-linked proteins, the SCD reveals a characteristic “halo” dispersed DNA loop, with no or minor halos indicating damage of DNA [220]. The SCSA makes use of metachromatic acridine orange, since it fluoresces red and green after attaching to either single-stranded or double-stranded damaged DNA [218].

Numerous studies define clinical cut-off levels for SDF prediction of spontaneous or ART-associated pregnancy [236, 237]. However, no consensus exists on a particular cut-off number, a recent review of the literature indicates a cut-off of 20% may be able to distinguish among fertile as well as infertile males. By examining native sperm treated by swim-up or DGC (density gradient centrifugation), in addition to instances involving personal or donor oocytes, various SDF levels have been recorded for predicting conception in pregnancy that occurs naturally or in ART settings, including ICSI and/or IVF. In spite of the inconsistency of available research and the difficulties in setting impartial cut-off values aimed at pregnancy estimate and clinical usage of SDF assays, the TUNEL assay is widely used because of its accuracy and reliability [197]. However, in specific clinics, the choice of SDF assay is frequently influenced by factors such as instrument availability, qualified people, and the cost of reagents and run- time.

2.5.2.5 Correlation amongst different SDF assays

While direct assays like TUNEL and indirect assays like SCSA cannot be directly compared due to their distinct DNA damage detection processes, a correlation appears to be detected when utilising threshold values to distinguish people with high SDF from a control group [238]. Cui et al. found that SDF assays have great diagnostic accuracy in detecting infertility, by sensitivity of 80% and specificity of 83%, and 0.92 of AUC (area under the curve) [239]. The combined sensitivities of the SCD were 0.77 and of Comet assays was 0.91, with 0.91 and 0.84 of specificities, correspondingly, whereas TUNEL test given a combined specificity and sensitivity of 0.91 and 0.77. In associated to the Comet and SCD assays, the TUNEL approach detected and distinguished individuals with SDF from the control group with greater precision.

Even though there have only been a few studies that look at the link between different types of SDF assays, some significant findings have been published. Chohan et al. discovered a substantial association ($P<0.001$; $r > 0.866$) between the SCSA assay besides both the TUNEL and SCD assays in fertile as well as infertile individuals, however no relation with the AO test [240]. When defining SDF in patient and control donors, Garca-Peiro' et al. found strong relationships between the TUNEL and SCD with SCSA assays [241]. Ribas-Maynou et al. performed a thorough investigation using multiple SDF assays and discovered a robust association amid SCSA and TUNEL, SCD and TUNEL, and SCD and SCSA [242]. There was an adequate connection between the assays as SCD and alkaline Comet, SCSA and alkaline Comet, and TUNEL and alkaline Comet. However, no association between other assays and neutral Comet assay is observed. In couples enduring ART, Simon et al. [243], discovered positive connection amongst the TUNEL and Comet. The SCSA and TUNEL assays produced identical outcomes for SDF in infertility patients than the control group, and a good connection was seen between the SCSA and TUNEL assays.

2.5.2.6 Difference between all SDF assays

Inter-observer besides inter-laboratory difference associated with known methodologies has hampered the commercialization of SDF test. Table 3 explained the all tests have set off limits and disadvantages [201]. Although the SCSA test shows the lowermost variation in intra-laboratory and inter-laboratory, the technique is not yet commercialised. Due to inter-observer inconsistency, other assays CMA3 staining, AO, SCD testing and toluidine blue staining face difficulties. However, when compared to the SCSA approach, the TUNEL assay has minimised variation by protocol standardisation, resulting in minimal inter-laboratory variability [244].

To address these concerns, ten laboratories from the Florence collaboration collaborated to standardise the technique for TUNEL, Comet, and SCSA assays. These labs examined the identical samples set to assess the degree of correlation between these assessments and the extent of difference between the labs. The goal of this programme was to increase the consistency and reliability of SDF testing in a variety of contexts.

Table 3: Techniques of sperm DNA fragmentation

Sr. No.	Technique	Drawback	Benefit
1	SCSA	• Needs expert technicians • Costly equipment like flow cytometer	• Reliable count of DNA-damaged sperm percentage
2	Comet	• Needs expert and experienced evaluation • Inconsistency in Inter-observer	• Needs very little sperm count. • Subtle • Reproducible
3	TUNEL	• Needs lab tuning	• Reliable, Sensitive • Results negligible inconsistency among interobserver • Needs few sperm
4	SCD	• Inconsistency in Inter-observer	• Simple test

The sperm DNA fragmentation measurement using various techniques has numerous drawbacks and lacks standardised clinical reference values, which has led to debates about their frequent usage. The SCSA test is regarded a straightforward indirect approach among the existing SDF assays, however it still has drawbacks that prevent its broad use and commercialization. TUNEL, on the other hand, is a direct assay that provides more accuracy in measuring SDF. The optimisation and standardisation of the widely applied TUNEL assay have reduced variation for intra-laboratory results, resulting in higher positive predictive value with more precise clinical SDF testing. This discovery improves the comprehension about infertility's fundamental causes. While the application of SDF assays in clinical practise is still

in its early stages, future large-scale research has the potential to broaden the scope of testing of SDF for infertile duos, resulting in improved management and treatment options.

2.5.2.7 Advantages of SCD over other SDF techniques

In the investigation of SDF, the SCD test has benefits over other assays like Comet, SCSA or TUNEL [245]. The ability to immediately visualise DNA fragmentation in individual sperm cells is one gain of the SCD test. It entails the elimination of DNA-linked proteins, which causes the chromatin loops to disperse. Under a microscope, this distributed DNA pattern appears to be seen, allowing for visual fragmentation assessment in DNA. Furthermore, unlike the Comet and TUNEL assays, the SCD test does not require DNA denaturation. The Comet assay detects DNA damage via electrophoretic separation of fragmented DNA, whereas the TUNEL assay marks DNA strand breaks. These extra steps in the Comet and TUNEL assays could introduce unpredictability and artefacts. The SCD test also has strong inter-laboratory repeatability showing the consistent results across laboratories. This is critical for developing standardised techniques and guaranteeing consistent and comparable outcomes in SDF analysis.

Chapter 3

Hypothesis

3.1 Introduction

Male infertility is recognized as a complex as well as multifaceted condition. In recent years, it has become more prevalent around the world. However, it is a serious health problem impacting nearly 8–12% of couples globally. The diagnosis of male infertility is typically performed through sperm analysis, which assesses various physical and microscopic factors, including sperm count, motility, and morphology. Though there are several confines to sperm analysis as the anomalies in these features do not correlate each time with the fundamental cause of infertility. Hence, there is a need for improved diagnostic techniques, for instance, DNA fragmentation to scrutinise the molecular features of sperm quality. Also, these tests appears to deliver additional information about the functional aspects in addition to the molecular aspects of sperm quality.

Various risk factors have a considerable influence on the functional and molecular components of sperm quality, including lifestyle, age, BMI, as well as environmental and genetic factors. Lifestyle variables are risk factors for male infertility that include smoking, alcohol intake and drug abuse with obesity factor. Environmental factors appear to also affect male fertility due to the impact of pesticides, heavy metals, and radiation exposure. In addition, genetic factors like chromosomal abnormalities, gene mutations, and epigenetic alterations may cause infertility. These risk factors appear to cause oxidative stress, inflammation, and DNA damage in sperm, which contribute to impaired sperm quality and infertility.

According to a study, diet patterns appear to affect sperm quality based on macronutrients and calorie intake. The quantitative and qualitative features of a diet appear to negatively impact sperm DNA damage, motility, and vitality. Therefore, it is important to study dietary patterns to better understand these effects. Vegetables and fruits in a diet appears to reduce these negative effects due to their antioxidant molecules. On the other hand, a vegetarian diet may reduce sperm concentration and motility, and its consequences on male infertility need further study. This may be due to chemical residues or estrogenic compounds in the diet that have an adverse effect on semen parameters.

Fatty acids and antioxidants play an important role in spermatogenesis, in addition to the chemicals included in the process of food production, conservation, transport, and taste enrichment. Studies show that non-Mediterranean countries with high consumption of vegetables, fruits, whole grains, pulses, and fish have better sperm quality compared to people

consuming Western diets such as red and processed meat with refined grains, high-energy drinks, and sweets. Western diet patterns are allied with decreased semen quality, while vegetarian diet patterns are most related to better semen quality according to an extensive range of research in Europe, North America, East Asia, and the Middle East.

According to Jensen et al., addiction to caffeine affects semen quality. Adequate caffeine intake does not show much effect; however, heavy doses of caffeine affect normal morphology and sperm count. It has also been reported that 50% of total caffeine enters our body from coffee alone and 20% from cola. Men who drink cola have poorer semen quality, high or low BMI, are mostly smokers, and have reproductive disease compared to men who did not. Green tea caffeine has also been shown to lower levels of testosterone, FSH, and LH, which appears to lead to a decrease in testicular size and reduced sperm motility [118].

In addition, drinking water tainted with lead and cadmium appears to harm the reproductive system of men. It reduces semen quality by triggering hypothalamic-c-pituitary axis disruption or spermatogenesis due to contaminated water. Filtered water appears to reduce the risk of water intake containing heavy metals, as well as chemicals like pesticides and industrial chemicals. This study aims to examine the presence of risk variables and their impact on the functional and molecular components of sperm quality in infertile males. To collect relevant information from participants, a questionnaire will be administered before sample collection. Semen samples will be collected, stored, and maintained in accordance with WHO guidelines at the Andrology lab of the Progenesis Test tube baby centre. The physical and microscopic properties of the sperm will be evaluated following these guidelines. Performing the Fructose test is crucial as it indicates the level of spermatogenesis. This test will also be used to address Azoospermia, which is characterized by the nonappearance of sperm in the ejaculate. A change in the colour of the solution indicates a positive result for Azoospermia. Moreover, the SCD test will be conducted to assess DNA fragmentation in addition to evaluating physical and microscopic characteristics.

The study is significant since it has the prospective to recognize modifiable risk factors for male infertility in addition to improving treatment outcomes. Healthcare experts appear to deliver tailored interventions to raise male fertility by understanding how factors of age, BMI, and lifestyle affect the functional as well as molecular elements of sperm quality. Moreover, this study appears to lead to the expansion of new diagnostic and therapeutic choices for treating male infertility, refining the quality of life for infertile couples. Scrutinizing several

risk factors on the functional and molecular features of sperm quality linked to male infertility appears to deliver useful insights into the aetiology of male infertility as well as aid in the improvement of viable treatments. The research results may improve the quality of life of infertile couples and lead to novel male infertility diagnostic and treatment methods.

3.2 Method for accomplishing hypothesis

We will collect semen samples and retain them in the Femacre Fertility centre's Andrology lab for this study. Furthermore, physical, and microscopic criteria, including sperm numbers, motile sperm, morphology, as well as viability, will be assessed using the samples. The SCD test will be executed to assess DNA damage. Moreover, a questionnaire will be provided to participants to link the risk factors and results of the semen analysis.

The study will comprise male volunteers between the ages of 18 and 45 who are seeking infertility treatment at Femcare Fertility Centre. These individuals will be separated into two groups based on male infertility risk variables. A questionnaire will be utilized to assess the risk factors, and individuals will be placed in the risk factor group according to one or more risk factors. Conducting a statistical analysis, we will compare the physical and microscopic parameters with DNA fragmentation between the two groups. The study will highlight a number of risk factors associated with the severity of sperm abnormalities.

A vital diagnostic tool, semen analysis, appears to determine male reproductive health as well as fertility potential. Physical measures in the analysis provide a complete picture of the semen quality since the microscopic data appears to analyse the specific properties of the sperm. The physical parameters encompassed in the analysis are liquefaction time, colour, volume, and pH, while microscopic parameters embrace sperm count, motility, and morphology in addition to round cell and leukocyte concentration.

The time required for semen to convert from a gel-like condition to a liquid state is referred to as the liquefaction time. A protracted liquefaction time may indicate infection, inflammation, or obstruction in the reproductive system. Semen appears to range in colour from clear to yellow, and any deviation from the normal colour appears to suggest infection or other health difficulties. The volume of ejaculated semen is measured in millilitres, and a lesser volume than usual may suggest a problem with the accessory glands that make semen. The pH of sperm

is usually alkaline, and any variation from this range appears to suggest problems with the prostate or seminal vesicles.

The most important indications of male fertility are the microscopic parameters of sperm examination. The sperm count, or the quantity of sperm per millilitre of semen, is an important component in male fertility. A low sperm count appears to indicate several health problems, including hormone imbalances, infections, and genetic abnormalities. The capacity of sperm to move and swim effectively is referred to as motility. Many reasons appear to contribute to poor motility, including inflammation, infection, hormone imbalances, and oxidative stress. The size and shape of individual sperm cells are referred to as the morphology of sperm, and aberrant morphology appears to signal genetic problems or other health issues. Round cells and leukocytes are not normally found in sperm, but their presence may signify infection or inflammation.

3.3 Expected outcomes from the study

The samples are necessary for analysing various variables based on the questionnaire. Furthermore, the semen analysis provides information on the quality of the sperm. We anticipate that the existence of risk factors for male infertility will have a detrimental impact on the functional and molecular features of sperm quality, as measured by physical and microscopic parameters and the SCD test. Individuals with risk factors are likely to exhibit reduced sperm count, motility, and morphology, increased DNA fragmentation, and lower sperm viability compared to those without risk factors. Additionally, we expect a significant correlation between the number of risk factors and the extent of sperm abnormalities, suggesting that participants with a higher number of risk factors are more likely to experience more severe sperm abnormalities.

Semen analysis gives valuable information on male sexual health as well as potential fertility, and this work has crucial implications for infertility in men diagnosis and therapy. In the perspective of our study exploring the impact of risk factors like age, BMI, and lifestyle factors on semen quality, it is important to discover potential patterns or links between the physical and microscopic features of semen analysis and the risk factors. Healthcare providers appears to utilize the analysis to determine potential health conditions affecting semen quality associated with male fertility.

This study focuses on the influence of several factors on functional and molecular components of semen quality that appears to help healthcare providers build personalised treatment programmes intended for individuals with male infertility. Also, the findings of our study may highlight the fundamental causes of infertility in males, helping in the creation of novel diagnostic tools as well as therapeutic strategies. Consequently, our research could help encourage the treatment of male infertility as well as avert issues related to infertility.

Chapter 4

Methods and Materials

Increasing issues in male infertility across the world are affecting several couples. Consequently, it is significant to focus on various factors associated with male infertility. There are several factors like lifestyle, age, and medical conditions affecting the semen quality which is associated with the finding of male infertility as well as the action. However, studying these factors appears to result in valuable insights for the diagnosis besides treatment for infertility in men. Our aim is to investigate semen quality for functional and molecular aspects of various risk factors in male infertility. The participants included in the study are diagnosed with male infertility and seeking treatment at a fertility clinic. These participants were carefully chosen according to the inclusion criteria. Subjects between the ages of 18 and 45 who have been trying to conceive for at least a year without success are considered in the investigation. Exclusion criteria are determined for men with a history of genital tract infections in previous 3 months, surgery, or trauma, antipsychotic drugs user and non-consenting cases.

An SPSS software is used to analyse the data collected through the questionnaire. To test the hypothesis, inferential statistics will be used after summarizing the data with descriptive statistics. The outcomes of the study will contribute to an enhanced understanding of the effect of diverse risk influences on semen quality related to male infertility. Moreover, it could inform the improvement of new diagnostic and treatment approaches. Whole study is differentiated into how Age, BMI, lifestyle (smoking, alcohol) impacts the functional parameters of semen quality. Our study also shown that how these factors affect the molecular parameters of semen quality. In present study we have also study the impact of medical history, diet and quality of drinking water quality of semen parameters. We have also observed the seasonal impact on semen parameters.

4.1 Study Design

This study was conducted using a cross-sectional study design to collect semen samples from male participants at the Femcare Fertility Centre in Pune, Maharashtra, India. The Figure-24 depicts the study design, which includes various components. It starts with the distribution of a questionnaire to collect relevant information. The collected data is analysed to determine any relationships or correlations. To ensure proper management of sperm samples, sample collecting and storage methods are followed. Figure 24 emphasises all two important factors including the functional features of semen quality and the molecular aspects of DNA fragmentation. Functional features include sperm motility, concentration, morphology, and vitality, which are analysed for determining the quality of the sperm.

Figure 24: Process of data collection to analysis of semen quality

Furthermore, the molecular element focuses on DNA fragmentation analysis to identify the percentage of sperm DNA injury. This research sheds light on the integrity and stability of the gene contained within sperm cells. Furthermore, the study emphasises the analysis of semen parameters, which entails assessing numerous semen quality traits and measurements. This research subsidizes to an improved knowledge of the overall reproductive potential of the sperm samples. Finally, the diagram shows that data analysis carried out to study the impression of risk factors on the semen quality allied with male infertility. This stage seeks to discover any characteristics or variables that may influence sperm quality and so contribute to male infertility or reproductive difficulties.

4.2 Materials used

Materials used in the research on the influence of various risk factors on functional and molecular aspects of semen quality associated with male infertility included:

1. ART lab: A well-equipped and maintained ART lab (assisted reproductive technology) was used for semen collection, storage, and analysis. The lab was equipped with all the necessary tools and instruments required for semen analysis, including a Laminar air flow, microscope, centrifuge, pipettes and Maklers chamber etc
2. Semen samples: Humanoid semen samples were collected from a sample population consisting of men with non-pregnant female partners. A total 1,006 participants were included in semen samples collection.
3. DFI kits: DFI kits were included in the DNA fragmentation evaluation in the collected semen. A total analysed semen samples for DNA fragmentation were.
4. SPSS software: A software of Statistical Package for Social Sciences was applied for data analysis and report compilation. The software was used to perform statistical analyses on the collected data and to generate graphs and charts for visualization of the results

4.3 Evaluating the impact of the factors on functional parameters of semen

The materials employed in the study investigating the impact of various risk factors on the functional aspects of semen quality were, an assisted reproductive technology lab (ART lab), semen samples, and SPSS software. An ART lab, meticulously maintained and well-equipped, plays a crucial role in the collection, storage, and analysis of semen samples. The

lab contains all the essential equipment's and instruments necessary for semen analysis, including a microscope, laminar airflow, centrifuge, cryo-tank, micropipettes and refrigerator etc. as well as disposables like sterile semen collection containers, glass slide and microslide etc. The human semen samples were sourced from a study population comprising a total of 1,006 participants. For the purpose of data analysis and report generation, Statistical Package for Social Sciences software (SPSS) was utilized. This software conducts rigorous statistical analyses on the collected data and produces visual aids such as graphs and charts to facilitate result interpretation. Every material utilized in the research was cautiously selected and designed to ensure the utmost accuracy and reliability of the findings. The integration of a well-equipped ART lab, specialized analysis tools, and IBM SPSS Statistics software, Inc., version 20.0 (IBM Corp., Armonk, NY, USA) enables a comprehensive analysis of the collected semen samples and the assessment of diverse risk factors impacting semen quality. The statistical inference employed two-sided tests with a significance as level of 0.05. Quantitative variables of semen quality were presented as mean values with standard deviation, indicating the significance as level of the analysis. The statistical analysis includes vivid statistics of semen parameters, correlation analysis and factor analysis with semen volume, progressive motility (PR), motility rate, and total sperm count. It describes the relationship between semen parameters and risk factors. The parameters were then compared with demographic and medical attributes, such as age, smoking, tobacco use, alcohol consumption, infertility status.

4.3.1 Sampling procedure

The participants were selected using a convenient sampling method. The study included men between the ages of 18 yrs to 55 years who were willing to provide semen samples for analysis. The participants included in the study were diagnosed with male infertility and sought treatment at a fertility clinic (Femcare Fertility Centre in Pune, Maharashtra, India). These participants were carefully chosen according to the inclusion criteria. Men of 20 to 55 years of ages, trying for conceiving from more than one year without success, were included in the study. A total of 1042 men, experiencing fertility issues, were recruited between 2021-2024 among them 36 were excluded as per exclusion criteria. Exclusion criteria were determined for men with a history of genital tract infections, surgery, or trauma. Exclusion criteria included long-term chronic illnesses, a past of thyroid-related syndromes, reproductive system trauma and surgery, sexually conveyed illnesses, genitourinary system

diseases, occupations with potential exposure hazards, and a history of exposing to toxic substances causing high-risk in reproductive system.

4.3.2 Place of Data Collection

The data for present research were acquired from the Femcare Fertility Centre, Pune, Maharashtra, India. Prior permission was obtained from the Centre to access the data. A questionnaire was prepared to capture all the relevant information before sample collection. The questionnaire included questions on demographic characteristics, medical history, lifestyle habits, and sexual behaviour. The questionnaire was administered to the participants before semen collection. The collection, storage, and maintenance of the semen samples were accomplished in harmony with the norms outlined by the WHO (fifth edition). These procedures were carried out in the Andrology Lab. of the Femcare Fertility Centre, ensuring adherence to the recommended guidelines to maintain sample quality and integrity. Participants were provided with detailed information about the study in addition to the written consents from them. The questionnaire focused on demographic characteristics like age, education, occupation, and personal habits such as smoking, alcohol consumption, and chewing tobacco. Additionally, participants were assessed for infertility type and prior surgeries, and provided with a semen analysis report. analysis and were separated as high score, medium score and low score groups based on semen quality.

4.3.3 Physical inspection of subjects

All participants underwent a physical inspection and interview conducted by an approved physician. The examination comprised measurement of height in addition to weight, BMI calculation, palpation, and urogenital visual check. Various factors were included in the interview such as drinking status, duration of abstinence, residence place, intercourse frequency, educational information, life evaluation, personal data, and history of current medication.

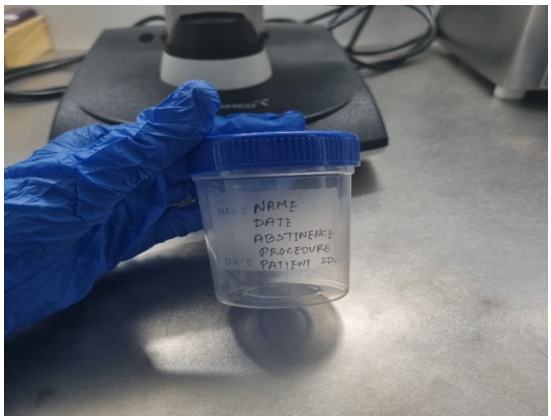
4.3.4 Methodology used for analysis of functional parameters

Semen samples for functional parameters analysis were collected from 1006 patients. Sample collection was done following the WHO manual (2021). Semen samples were collected using the standard procedures suggested by the WHO [28]. The samples were collected through masturbation next to 2-7 days of abstinence period. The samples were gathered in sterile collection flasks and will be transported to the Andrology Lab. of the Femcare Fertility Centre for further analysis. Each male participant's semen sample was obtained, preserved in a sterilized container of plastic, and promptly sent to the test site for investigation. A competent embryologist measured sperm concentration, sperm volume, total sperm number, including the motility of sperm according to WHO norms. Following the entire liquefaction of the specimens, standard semen analysis took place. Professional embryologist examined sperm samples, and the mean of their measures was utilised for each reading.

Liquefaction of samples was performed immediately following collection, after which microscopic analysis of semen parameters was undertaken, which included semen volume (ml), sperm count (M/ml), total motility (TM) of spermatozoa (%), progressive motility (PR) of spermatozoa (%) and morphology of spermatozoa (%). Semen volume (ml) measurement was performed by providing patients with a measuring container, from which data was recorded. Sperm count (M/ml) was calculated by using standard Makler counting chamber (Sefi Laboratories, Tel Aviv, Israel), for which 10 microliter of well-mixed semen sample was micropipette on the Makler Chamber and was observed under 20X magnification of a light microscope. Figure 25 shows the procedure and tools used to evaluate the parameters like count, motility and morphology etc.

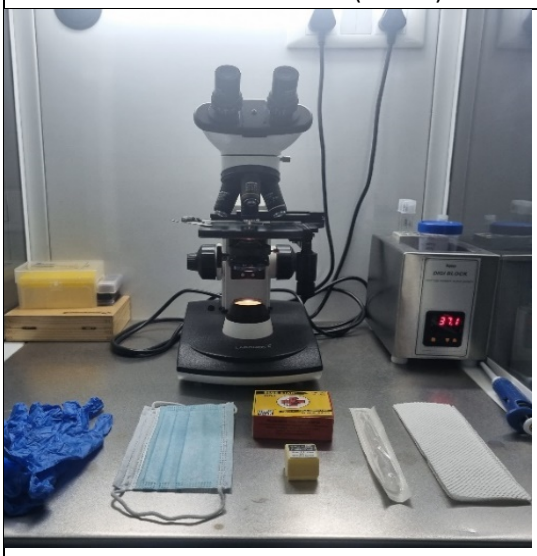
Sperm motility in TM (%) and PR (%) was observed by making a wet preparation of 10 microliters of well-mixed semen sample on a glass slide, which was then observed under 40X magnification using light microscopy. A minimum of 200 spermatozoa were observed to calculate the percentage of motile sperms among total number of sperms. Morphology of spermatozoa were determined by Diff-Quik staining procedure, from which resulting slides

were observed under oil immersion objective of 100X magnification as per Kruger criteria. A minimum of 200 spermatozoa were evaluated.



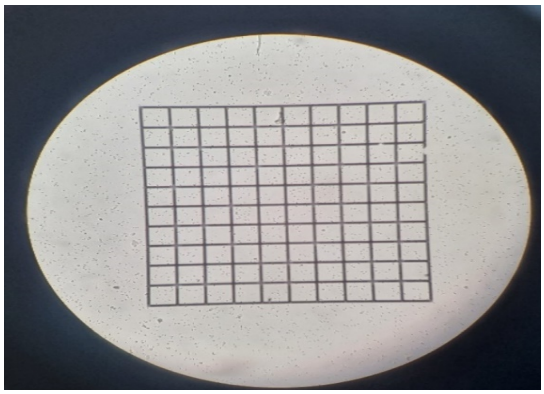
a. Semen collection container (Tarson)

b. Makler chamber (sefi, israil)

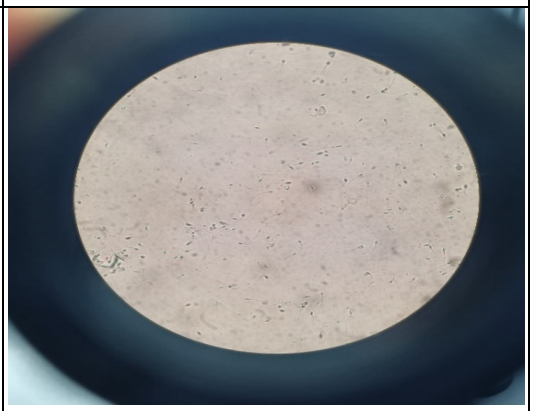


c. LAF with, Binocular Microscope (Magnus), Glass slide (Blue star), dropper (BD)

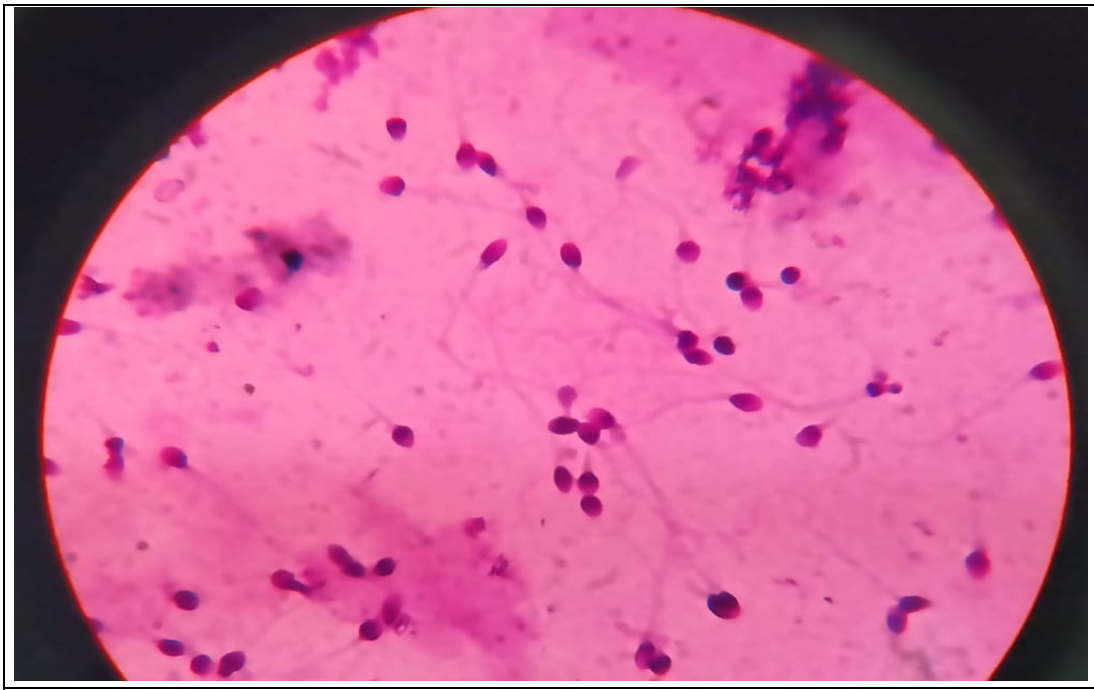
d. Centrifuge machine (REMI)



e. Grids of makler chamber showing 100 squires



f. Spermatozoa under 10X objective of light microscope



g. Morphology of spermatozoa under 100X oil immersion objective

Figure 25: Tools and procedure used in assessment of functional parameter of semen

The patients were also provided with questionnaires, which they were required to fill out. including information about the lifestyle, diet, physical condition, and medical history of the participants. The questionnaire focused on physical conditions, including age, BMI, medical history, medications, stress, family history, and lifestyle influences, like frequency of smoking, tobacco chewing and alcohol consumption, drug use, tea-coffee intake, filtered water use, diet patterns, and physical activity. Medical conditions, such as diabetes, surgery, and hypertension, were also included, as well as exposure to chemicals, pesticides, etc. the body weight of the patients along with their height was also recorded at the time of sample collection for calculating their BMI. Additionally, the participants were categorized by level of consumption, to investigate the impact of all factors on semen quality. All collected data were summarized as mean ± standard deviation (SD) and then categorized into different groups based on their respective frequencies. Groups were compared by one-way analysis of variance (ANOVA) Pearson correlation analysis and $p < 0.05$ was considered to be statistically significant. Pearson correlation analysis was undertaken to assess the association between variables.

4.3.5 Grouping of all factors

Age factor: In order to analysed the impact of age on semen quality, Semen samples are distributed across six ages grouped, group-I (<25 years), group-II (26-30 years), group-III (31-35 years), group-IV (36-40 years), group-V (41-45 years), and group-VI (>45 years), facilitating parameter evaluation.

Obesity factor: BMI groups are established as underweight (<18.5 kg/m^2), normal (18.5–24.9 kg/m^2), overweight (25–29.9 kg/m^2), and obese (≥30 kg/m^2), enabling the observation of obesity's impact on semen quality.

Lifestyle factor: These factors including different subfactors like unhealthy habits including Tobacco consumption, Cigarette smoking, alcohol consumption

Smoking habits: the impact of cigarette smoking on semen parameters were analyzed by dividing the subjects initially into two groups group (group-I nonsmoker, group-II was smoker) and again subjects characterized into total four groups according to their smoking frequency Group-I (0 cigarettes per day), Group-II (1-2 cigarettes per day), Group-III (3-4 cigarettes per day) and Group IV (>5 cigarettes per day) consumption.

Consumption of tobacco: the impact of tobacco consumption on semen parameters were analyzed by dividing the subjects initially into two groups group (group-I no consumer, group-II consumer) and again subjects characterized into total four groups according to their tobacco frequency. Group-I (o time i.e. non consumer), Group-II (1-5 times per day), Group-III (6-10 times per day) and Group IV (>10 times per day).

Alcohol intake: to evaluate the impact of alcohol on semen parameters subjects were categorize into Group-I (non-alcoholic), Group-II occasional drinker (1-3 times/15 days), Group-III regular drinker (4-10 times/ 15 days) and Group-IV addicted (>10 times/15 days) groups.

Type of diet: Influence of Veg and Non veg diet on semen quality were studied by comparing and categorizing the semen samples into two groups. Group I was the vegetarian in their diet whereas Group II was the group of non-vegetarian diet.

Medical history: all subjects were categorised into two categories. Group first saying "NO" was don't have any serous past medical history. Whereas second group saying "YES" had specific health related serious concern it may be disease, surgery, accident or any chronic heath related issue.

Type of drinking water: For the analysis of functional aspects of semen quality the participants have been divided into two groups based on their use of filtered water (Group-I) or unfiltered water (Group-II) for day-today drinking.

Consumption of Fast Food: to evaluate the impact of fast-food consumption on semen parameters subjects were categorised into two categories. Group first saying "NO" was not eat fast like burger, pizza, samosa etc. Whereas second group saying "YES" had regular eaten the fast food.

Food Supplement: For the analysis of impact of extra food supplement such as protein powder, protein tablet, immunity buster etc on functional aspects of semen quality the participants have been divided into two groups Non consumer (Group-I) and Consumer (Group-II).

Tea/coffee (caffeine intake) consumption: An impact of tea or coffee consumption on semen parameters were observed in by dividing subjects into two groups: one consisting of individuals consuming tea/coffee and the second not consuming tea/coffee.

Fizzy Drink consumption: Consumption of non-alcoholic carbonated beverages contain dissolved CO_2 was investigated for its impact on semen parameters by categorizing the samples into two groups. Group I is non consumer of fizzy drink and Group-II was consumer of fizzy drink.

Exposer of Chemicals and Pesticide: Subjects who exposed to chemicals and pesticide are characterised into two groups to evaluate their impact semen quality as Group-I (non-exposed group) and Group-II (exposed group).

4.4 Evaluating the factors impact on DNA fragmentation by using SCD method

Conventional semen analysis was performed on all semen samples as it was a basis test to evaluate the semen quality, whereas DFI was advise only those patients who has repeatedly abortion history, multiple IVF failure, multiple intra uterine insemination (IUI). To address the issues in ART, a new diagnostic tool of SCD (see figure 26) tests for SDF has been introduced [246]. The test looks for the presence of a distinctive halo of distributed DNA loops, which is missing in sperm DNA with fragmentation next to acid denaturation including nuclear protein elimination [220]. The results are correlated strongly with the SCSA (sperm chromatin structure assay), an influential tool routinely used on samples of human sperm. Furthermore, the test is easy, inexpensive, and produces results quickly [219]. To examine and compare conventional sperm parameters with sperm chromatin fidelity in releases from men with partners having an RPL history (recurrent pregnancy loss) besides a control group of healthy men using SCD test.

Lower percentages of normal sperm morphology during IVF cycles are connected with lower rates of effective fertilisation and pregnancy per cycle, as well as an augmented risk of failure even after fruitful embryo transfer [247]. Greater rates of SDF is discovered in couples who experienced recurrent initial pregnancy loss after impulsive conception [248]. According to Borini et al., greater rates of initial pregnancy loss is seen in couples undertaking ART like IVF as well as ICSI in case of elevated sperm DNA fragmentation and aberrant morphology [249]. However, male factors such as genetic factors, sperm parameters, and sperm morphology may contribute to recurrent pregnancy loss [250].

Furthermore, increased DNA stainability and fragmentation have been associated to poor sperm morphology and RPL. A current work found that abnormalities in structure of sperm DNA, such as high fragmentation of DNA with stainability, not indicated significant association with ICSI or IVF rates, quality of embryo, or rates of pregnancy, however it is associated with higher rates of spontaneous abortion after implantation [251]. As the number of infertile couples seeking ART rises, there was increased worry regarding fertility potential reduction and the male factors' role in recurrent loss of pregnancy.

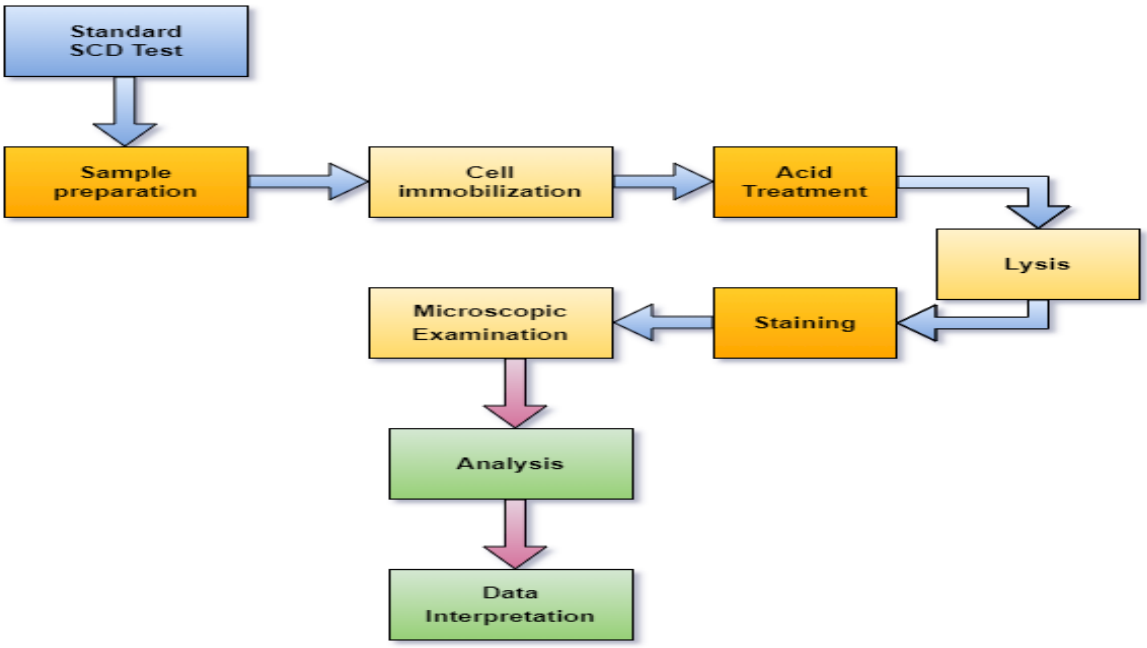


Figure 26: Process of SCD test for semen parameters' analysis

4.4.1 Material used

We have performed sperm chromatin dispersion (SCD) method for evaluating the percentage of DNA damage in semen samples. For performing SCD method DNA fragmenting well equipped andrology lab, LAF, dropper, light microscope, refrigerator was required. DFI (%) test was performed by using commercially available Sperm Chroma kit (CryoLAB International, India). Which included precoated glass slide, agarose gel, four different solution-A (denaturing agent), solution-B (lysis solution), solution-C (eosin stain), solution-D (thiazine stain) and Three types of dehydrants in increasing order (70%-100%). Additionally, mini-incubator was also required for melting the agarose.

4.4.2 Methodology and Procedure

Semen sample for analysis of molecular parameter were collected from 530 subjects who visited our ART clinic. Sample collection and conventional semen analysis was done as per section of 4.3.4**,** Additionally, sample were procced for evaluating the DNA fragmentation. Agarose Gel was melted by using mini-incubator for five minutes at 90^{O}C and subsequently at 37^{O}C). Added

25µl of semen sample in agarose gel and mixed it well. Placed 10 µl drop of this sample on a precoated glass slide and covered it with cover slip by avoiding the formation of any air bubble. Slide were kept at $4^{O}C$ (to solidify the agarose with the spermatozoa embedded within) on cold surface for five minutes. carefully removed the coverslip by gently sliding it off and kept the whole slide horizontally to apply denaturing agent for seven minutes. Removed out denaturant agent without any shaking. After that applied lysis solution for 20 minutes. Washed this slide with abundant of distilled water for 5 minutes and then applied dehydrants in increasing order (70%-100%) with interval of 2 minutes of each. Slide then stained with mixture of eosin stain and thiazine stain (5 drops of each) for 15-20 minutes. Decanted the excess stain and washed it gently with distilled water and dried at room temperature. Observation was done under 100X of light microscope and calculated minimum 200 spermatozoa for each sample. Measurement was done twice for accuracy. A positive control was run by lacking the addition of the denaturant agent, where all spermatozoa were shown with halo. A negative control was run, by lacking of lysis reagents where all spermatozoa lacked halo. Detail procedure explained in appendice-3.

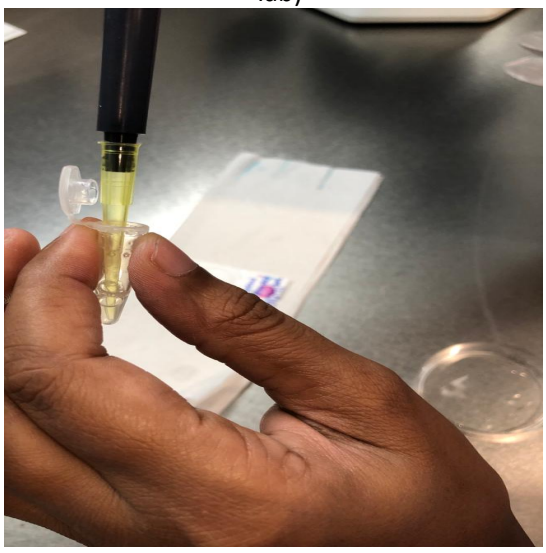
4.4.3 Patient counselling

The patients were also had interaction session for questionnaires, which they were required to confirm the details. Questions were asked as per Annexure -2.2 and data were maintained in excel sheet. including information about the lifestyle, diet, physical condition, and medical history of the participants. The questionnaire mainly included the question related with physical health, medical history, family history, and lifestyle influences etc. Once Questionnaire and SCD test completed on the basis of that subjects were characterised into different groups to evaluate the impact of different factors on DNA fragmentation. All groups the preparations of Age, BMI, Medical history, lifestyle factors (Cigarette Smoking, Tobacco and Alcohol consumption), Type of drinking water, Type of Diet, Fast food, Food supplement, Caffeine intake, Fizzy drink consumption, pesticide-chemical exposure and the statical analysis were carried out as per section 4.3.5 Thus, assessing damage in sperm DNA is useful as a standard test in infertility studies. Traditional bright-field microscopy appears to be widely used for DNA assessment, and the SCD test has the potential to enable routine screening of sperm DNA damage in basic andrology. Procedure of SCD is shown in figure 27 with each step including

Croma kit, sampling, mixing, slide preparation, mixed sample, placing cover slip on slide at 4-degree, dehydration, and staining.

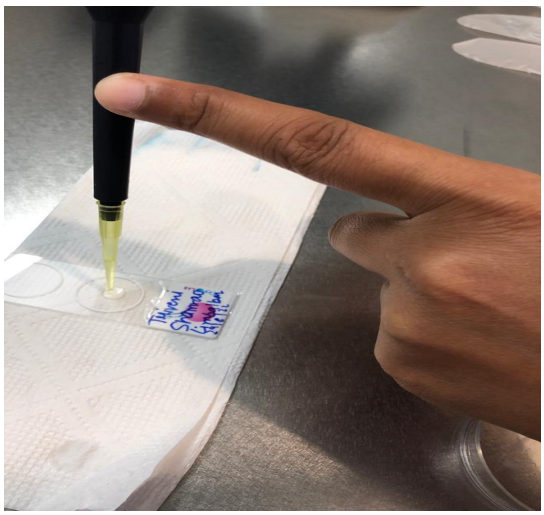
a) Solution a, b, c, d (Croma kit by cryo-lab)

b) Sampling

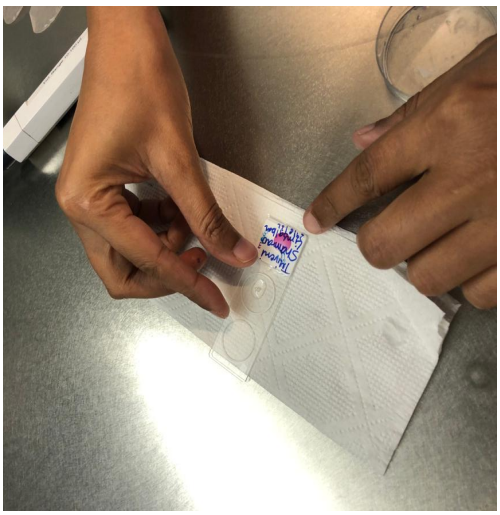


c) Mixing sample in melted agarose

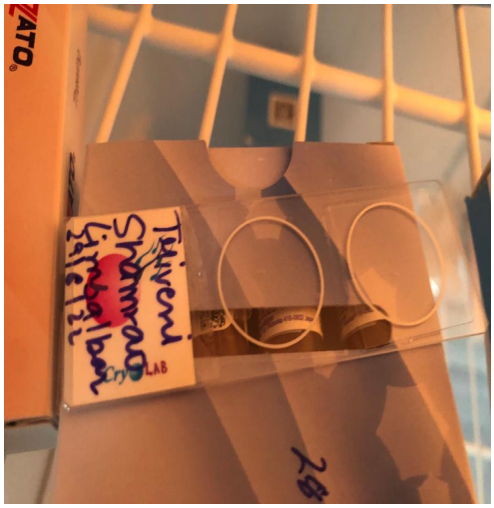
d) Slide preparation



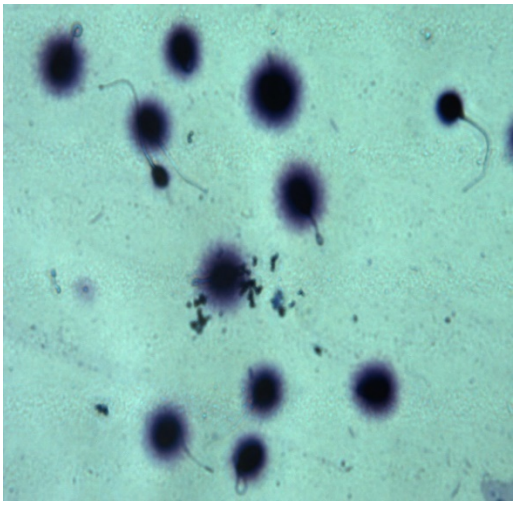
e) Mixed sample on pre-stained slide



f) Placing cover slip



g) Slide at 4 degrees



h) After dehydration of and staining

Figure 27: Procedure of SCD including a, b, c, d, e, f, g and h showing the different step of procedures,

4.4.4 Percentage of DNA Fragmentation by SCD Method

An evaluation of semen analysis between control group and recurrent pregnancy loss group reveals that semen volume and count fall within the standard range in both groups. However, there is an important reduction in sperm motility as well as morphology in the RPL group linked to the control group. To assess sperm DNA fragmentation, the SCD test is applied using conservative bright-field microscopy. The figure 28 identifies four SCD patterns of sperm cells

including a large-size, medium-size, and small-size halo, and without a halo. For large-size halos, the width is comparable to or greater than one-third of the minor length of the core. In the case of medium, the width falls between that of a high-size halo and a small halo, and for small-size halos it is either similar or smaller than the corresponding measurements.

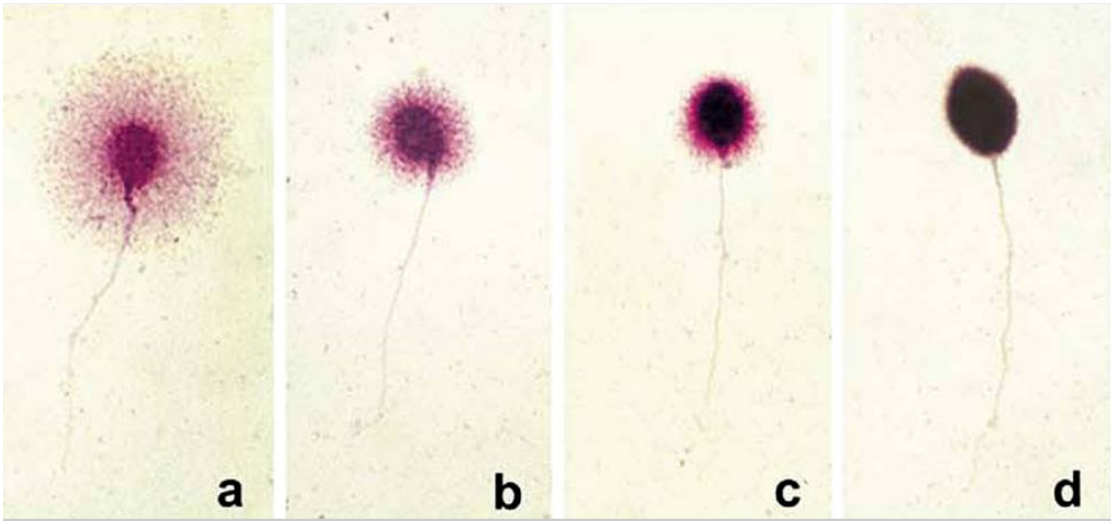


Figure 28: Halo analysis

Various investigations on infertile patients have produced conflicting results regarding the level of sperm DNA integrity. sperm quality investigated by Gopal k krishnan et al. in depth, focusing primarily on sperm nuclei and chromatin condensation. It found a correlation between repeated early pregnancy loss and low sperm quality, as evidenced by abnormal chromatin formation or an increase in sperm nuclear vacuoles [252]. People with recurrent pregnancy loss had significantly worse sperm functional test scores. A unique technique for measuring DNA fragmentation in human sperm cells, known as the SCD test, was recently introduced [251]. Although few research has investigated using the SCD test in cases of RPL, it has discovered that affected patients have a higher sperm cells percentage with a pronounced degree of nuclear dispersion. A good connection of the SCD test performed using conventional bright-field microscopy with the SCSA, proving its efficacy. The SCD test has evolved as a less expensive alternative to the SCSA, capable of distinguishing varying degrees of DNA fragmentation. Furthermore, this test detects severe DNA damage that may have a deleterious effect on nuclear structure and is not detectable using existing DNA damage assessment approaches [253].

4.4.5 Statistical analysis

SPSS is a software program referred to Statistical Package for the Social Sciences and widely applied to statistical analysis in social sciences [254]. In our research, the data obtained from SCD examination for Sperm DNA fragmentation was analysed using SPSS software.

The SPSS software allows researchers to conduct descriptive and inferential statistical analyses on data sets. For descriptive statistics, the software appears to generate frequency tables, Central tendency, and dispersion measures. These statistics appears to provide a summary of the data and assistance in classifying any patterns or outliers in the data. For inferential statistics, the software appears to conduct hypothesis testing, such as t-tests, ANOVA, and regression analysis, to obtain the meaning of the relationships amongst variables. In the case of the research on infertility in men, the SPSS software appears to be used to test the hypothesis that certain risk factors, like age, smoking, or exposure to chemicals, are related to augmented levels of Sperm DNA fragmentation.

The p-value is calculated using a t-test for semen analysis among two groups that represent the probability of two groups for no true difference in terms of the semen parameters being analysed. It indicates substantial proof over the null hypothesis, showing no difference between groups. A low p-value < 0.05 indicates that the semen parameter difference between groups is of statistical significance. There is evidence that the groups differed, thus the difference is unlikely to have been random. While a high p-value suggests weak evidence versus the null hypothesis. The observed semen characteristic difference is likely due to random variation, and there is insufficient information to conclude a substantial difference between the two groups. Thus, the significance as level and research hypothesis impacts the p-value's interpretation.

Semen analysis uses the correlation coefficient to determine connection strength and direction among variables within two groups. It measures linearity between variables. A correlation coefficient of -1 to 1 is possible. A correlation value of 1 shows an ideal positive linear relationship, where one variable rises proportionally to the other. The variables have a perfect negative linear relationship if the correlation coefficient is -1, suggesting that when one variable goes up, the other drops correspondingly. Stronger associations have correlation coefficients closer to -1 or 1. However, a 0-correlation coefficient implies no linear link.

Semen analysis uses a correlation coefficient to compare semen properties in two groups. Positive coefficients of correlation show that variables rise or decrease together, while negative correlation coefficients show the opposite. Remember that the correlation coefficient indicates linear relationships across factors rather than causality.

Statistical applications will be used to examine the information gathered from the questionnaire and semen analysis. The subjects' demographic traits and specimen characteristics will be compiled using descriptive statistics. The association between the semen parameters and lifestyle and demographic factors will be determined using inferential statistics. It is discussed in next chapter with results obtained from semen analysis and its relation with risk parameters.

4.5 Methodology used for evaluating the functional and molecular influence of SAP on semen quality

The samples from subjects were collected by masturbation. All semen samples were analysed according to the WHO andrology manual 2010. To evaluate the impact of sexual abstinence period (it was a period between two ejaculations) on semen quality, the collected samples were categorized according to sexual abstinence and segregated into four groups as Group-I (0 days abstinence) Group-II (1-03 days of abstinence), Group-III (04-06 days of abstinence), and Group-IV (07-10 days of abstinence), Group-V (>10 days of abstinence)

4.5.1 Evaluating Functional parameters

Macroscopic parameters such as volume and microscopic parameters such as Sperm count, Sperm motility and sperm morphology was analysed accordingly appendices-2. Sterile plastic containers were used for accumulating semen samples after abstaining from ejaculation for 2-7 days. The container should be non-toxic, non-pyrogenic, and non-spermicidal. It is essential to collect the samples close to the laboratory to minimize the time for analysis after collection. The samples endorsed to liquefy at 37 °C for thirty minutes and analysed in an hour of time for collection following the WHO 2010 criteria. After liquefaction, a small amount of the semen (around 6-10 µL) was in a Makler's counting chamber, and sperm count and motility were analysed under a bright field microscope at ×400 magnification. Minimum of 200 spermatozoa were assessed, and analysed with the average of duplicate measurements.

4.5.2 Evaluating Molecular aspects

DNA fragmentation percentage was evaluated by using SCD method with the help of commercially available kit. Standard protocol of SCD was used as per appendices-3. Statistical comparison was performed to compare values obtained between two groups. The significance as level was 0.05. Statistical analyses were performed with the help of SPSS. One way ANOVA was performed to look for significant differences in mean values between the different groups. A correlation analysis was performed to find out which marker was most successful in determining the underlying factors behind male infertility.

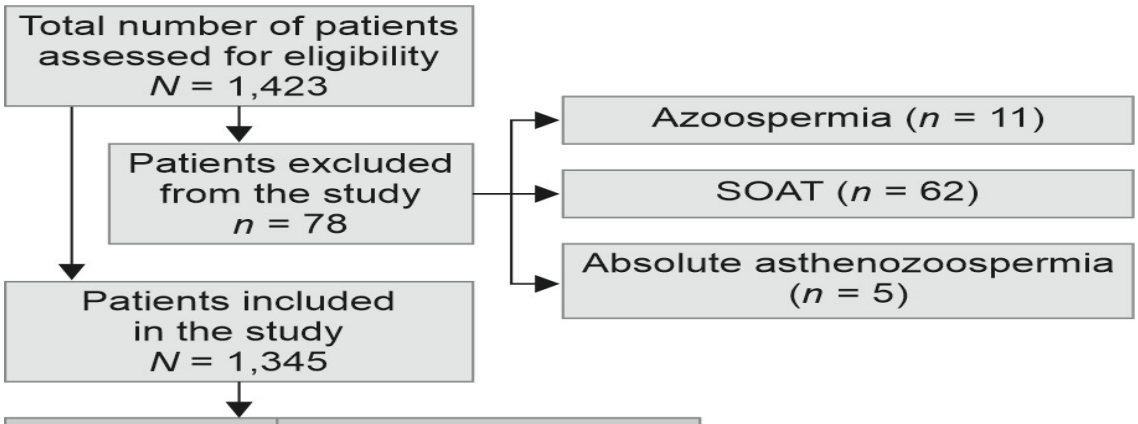
4.6 Seasonal impact on semen parameters

Study Setting and Materials This retrospective study was conducted at Femcare Fertility Centre, located in Pune, Maharashtra. Semen analysis report data were gathered from databases for all male patients of infertile couples who were examined at the centre over the 2-year period from March 2022 to March 2024. Each record included the patient's age, the date the sample was obtained, the duration of abstinence, and the semen analysis report. Data were grouped into four seasons according to the Indian Meteorological Department: summer (March–May), monsoon (June–September), autumn (October–December), and winter (January–February).7 Sampling Population A total of 1,423 subjects were analysed, of which 1,345 were included in this study. Patients with azoospermia, severeoligoasthenoteratozoospermia (SOAT), and absolute asthenozoospermia were excluded to avoid confounding factors.

Patients ages ranged between 21 and 50, and sexual abstinence of 2–7 days was maintained. Methodology and Laboratory Evaluation: Semen analysis was performed per WHO guidelines 2021 (6th edition). The semen sample from subjects was collected in a 100 mL sterile, clean plastic container (100 mL sample container, Tarsons, India) and was kept for liquefaction in a heating block (Ketan Digi Block, Shivani Scientific, India) at 37°C for 30–60 minutes. After liquefaction, a drop of 10 µL of the well-mixed sample was placed using a micropipette (Accupipette, Tarson, United States) onto a clean Makler chamber (Sefi Laboratories, Tel Aviv, Israel). The slide was then assessed under the microscope (Vision 2000, Labomed, United States).

4.6.1 Sampling Population

As per figure 29 total of 1,423 subjects were analysed, of which 1,345 were included in this study. Patients with azoospermia, SOAT, and absolute asthenozoospermia were excluded to avoid confounding factors. Patients ages ranged between 21 and 50, and sexual abstinence of 2–7 days was maintained



Seasons	Number of patients
Summer	269
Monsoon	406
Autumn	357
Winter	313

Figure 29: Number of participants selected after inclusion-exclusion criteria

4.6.2 Methodology and Laboratory Evaluation

Semen analysis was performed per WHO guidelines 2010. After collection of semen samples, it undergoes standard semen analysis and evaluation of microscopic and macroscopic parameters as mentioned in appendice-2

4.6.3 Statistical Analysis

IBM Statistical Package for the Social Sciences (SPSS) Statistics version 26.0 was used for statistical analysis. The patients were grouped into seasonal periods according to the date on which they visited the centre and the semen analysis was performed. Descriptive statistics of semen variables such as mean, range, and standard deviation were computed. In addition,

patients were given a particular impression based on their semen analysis report according to WHO criteria. A one-way analysis of variance (ANOVA) test was used to compare the mean values of semen parameters between seasons, and a paired Bonferroni post hoc comparison test was then used. A p-value of <0.05 was consider statistically significant and a p-value of <0.01 was consider statistically highly significant. Swarm plots and violin plots, which give a visual representation of the data distribution, were generated using the Seaborn library and Python programming.

4.7 Ethical considerations

Informed permission has been obtained from all contributors before sample collection. Confidentiality and privacy will be maintained throughout the study. The study was performed in harmony with the ethical guidelines and regulations set by the Institutional Ethics Committee (ECR/440/INST/MH/2013/RR-2016). Persons or couples experiencing infertility frequently pursue procedures with unknown effectiveness or questionable care because of the limits of current ART interventions and treatments. Surgical sperm retrieval for cases of excessive SDF or the use of anti-estrogens or gonadotrophins to treat nonobstructive azoospermia are two examples. Clinicians are challenged with competing ethical considerations when analysing these requests. In these instances, ethical issues often include autonomy, fairness, beneficence, and nonmaleficence. The choice to recommend or discourage an intervention is based on a mix of these criteria, which consider the quality of evidence supporting the intervention as well as the patient's unique characteristics. However, when confronted with unproven medical therapies in the realm of infertility, practitioners frequently face ethical quandaries.

A shared decision-making method is advised to overcome these quandaries and foster collaborative decision-making. This method entails the doctor offering detailed information regarding the medical elements of the intervention, outlining current best practises, and responding to any concerns or questions voiced by the patients [255]. Ethical concerns about unproven medical interventions appears to be addressed in a patient-centred and collaborative manner by using a shared decision-making approach, ensuring that the treatment strategy relies on the best available evidence as well as respects the values of autonomy, justice, beneficence, and nonmaleficence [256].

Chapter 5

Results, Discussion and Conclusions

In present research, humanoid semen samples were collected and analysed to investigate the functional and molecular aspects and their influence on semen quality. We examined various factors to evaluate the functional aspects such as, volume, sperm count, motility (total motile sperm and progressive motility), and morphology and molecular aspects such as DNA fragmentation.

The analysis involved physical aspects, such as colour, volume, pH, and microscopic aspects, including sperm count, motility (total motility and progressive motility), morphology, round cell count, and leukocyte count. Additionally, we conducted a questionnaire-based analysis to gather information on factors like age, BMI, medical history, environmental conditions, occupational factors, stress levels, diet, and lifestyle choices. These factors were assessed for their influence on sperm quality. To delve deeper into the data, we grouped the information based on BMI ranges and levels of tea/coffee consumption, alcohol, and tobacco use, among others. By doing so, we aimed to know the effect of these factors on semen quality.

Our study aimed to provide comprehensive insights into the relationship amongst various parameters, lifestyle variables, and semen quality. The results obtained will contribute to our understanding of how these factors affect male fertility and assist in formulating recommendations for individuals seeking to optimize their reproductive health. Based on the information presented below, we are examined the relationship between various factors and their influence on semen parameters and DNA fragmentation. Our analysis reveals different correlations among these factors. The correlations encompass a wide range of factors, which have been categorized as health factors, lifestyle factors, diet factors, SAP factors, and environmental factors. These categories encompass a comprehensive assessment of the different aspects that may impact semen quality and DNA fragmentation. We examine correlations to determine how these risk variables may affect male fertility health.

5.1 Impact of Factors on Functional parameters of semen

Semen parameter data were collected from 1,006 patients, and the results were analysed in relation to age, BMI, and lifestyle factors such as smoking, alcohol consumption, and tobacco chewing. The mean values and SDs of these parameters are shown in table no 4 whereas result of Pearson correlation analysis of same factors were mentioned in table no 5.

Table 4: Mean and SD values of semen analysis showing the impact of age, BMI, smoking, alcohol on functional parameter of semen

	No. of participants	Semen volume (mL)	Sperm count (M/mL)	TM (%)	PR (%)	Morphology (%)
Age range						
20–25 years	37	1.62 ± 0.69	28.27 ± 14.46	45.75 ± 18.79	25.51 ± 5.05	1.56 ± 0.92
26–30 years	220	2.05 ± 1.02	30.01 ± 17.36	43.8 ± 19.04	23.43 ± 4.84	1.61 ± 1.17
31–35 years	398	2.18 ± 1.48	31.53 ± 17.86	42.76 ± 18.53	22.96 ± 4.79	1.55 ± 0.96
36–40 years	217	2.17 ± 1.27	30.98 ± 18.44	40.13 ± 20.83	21.75 ± 4.66	1.54 ± 1.08
41–45 years	75	2.04 ± 1.7	34.01 ± 17.41	44.93 ± 17.98	24.09 ± 4.9	1.66 ± 0.96
>46 years	59	1.76 ± 1.39	37.05 ± 20.53	41.93 ± 22.29	23.79 ± 4.87	1.71 ± 1.14
	p-value	0.01	0.07	0.26	0.47	0.83
BMI range						
Underweight (<18.5)	55	2.23 ± 1.41	29.3 ± 17.52	40.56 ± 20.42	21.54 ± 13.1	1.38 ± 0.99
Normal weight (18.5–24.9)	422	2.03 ± 1.07	32.72 ± 17.65	45.1 ± 18.99	24.87 ± 12.67	1.74 ± 1.13
Overweight (25–29.9)	381	2.2 ± 1.26	32.45 ± 18.25	41.61 ± 19.14	22.08 ± 12.53	1.54 ± 0.97
Obese (≥30)	148	1.96 ± 0.93	26.16 ± 17.29	39.08 ± 20.01	20.78 ± 12.66	1.31 ± 0.89
	p-value	0.06	0.00	0.00	0.00	0.00
Smoking frequency (per day)						
Non-smoker	843	2.11 ± 1.18	32 ± 17.5	43.26 ± 19.09	23.39 ± 12.57	1.6 ± 1.05
1–5 times	138	2.03 ± 1.01	27.78 ± 19.67	38.74 ± 20.39	20.73 ± 13.2	1.45 ± 1.04
6–10 times	17	1.7 ± 0.92	29.29 ± 19.25	40.41 ± 23.94	22.17 ± 15.5	1.41 ± 0.79
>10 times	6	2.05 ± 0.38	47.16 ± 25.27	51.16 ± 17.09	27.33 ± 13	2.16 ± 1.32

	p-value	0.45	0.01	0.05	0.11	0.20
Tobacco chewing frequency (per day)						
Nonuser	770	2.12 ± 1.17	30.87 ± 17.65	42.15 ± 19.26	22.75 ± 12.69	1.56 ± 1.05
1–5 times	153	1.96 ± 1.12	34.47 ± 18.15	46.47 ± 18.92	25.81 ± 12.77	1.81 ± 1.12
6–10 times	65	1.99 ± 0.93	31.41 ± 19.77	40.95 ± 19.8	21.07 ± 11.89	1.38 ± 0.8
>10 times	18	2.31 ± 1.42	31.55 ± 20.91	37.55 ± 23.78	18.38 ± 13.82	1.05 ± 0.63
	p-value	0.29	0.16	0.04	0.01	0.00
Alcohol frequency (per 15 days)						
Nonalcoholic	628	2.12 ± 1.45	31.21 ± 17.2	42.28 ± 19.42	22.73 ± 12.76	1.58 ± 1.06
1–3 times	319	2.1 ± 1.44	31.59 ± 18.65	43.41 ± 19.08	23.64 ± 12.66	1.62 ± 1.04
4–10 times	35	1.83 ± 1.35	36.37 ± 20.58	44.51 ± 17.39	24.25 ± 11.4	1.48 ± 0.88
>10 times	24	1.75 ± 1.32	29.54 ± 22.98	39.29 ± 24.94	21.08 ± 14.53	1.16 ± 0.76
	p-value	0.23	0.38	0.62	0.57	0.00

*The age range of subjects was 20 to 55 years

Table 5: Result of Pearson correlation analysis

Correlation coefficient values					
	Semen volume (mL)	Sperm count (M/mL)	TM (%)	PR (%)	Morphology (%)
Age					
20–25 years	0.08	–0.03	–0.17	–0.11	–0.01
26–30 years	0.06	0.12	0.06	0.05	0.02
31–35 years	0.07	0.08	0.05	0.04	–0.06
36–40 years	–0.02	–0.04	0.02	–0.01	0.06
41–45 years	0.12	0.01	0.10	0.10	0.09

>46 years	–0.10	0.22	–0.08	0.01	0.11
BMI					
Underweight (<18.5)	0.05	0.05	0.06	0.00	0.08
Normal weight (18.5–24.9)	–0.17	–0.03	–0.01	–0.02	0.06
Overweight (25–29.9)	–0.04	0.02	–0.09	–0.05	0.05
Obese (≥30)	0.03	–0.13	–0.11	–0.22	–0.10
Smoking frequency (per day)					
Non-smoker	NA	NA	NA	NA	NA
1–5 times	–0.01	–0.19	–0.17	–0.17	–0.15
6–10 times	0.20	0.19	0.10	0.14	0.39
>10 times	–0.88	–0.24	0.31	0.36	–0.65
Tobacco chewing frequency (per day)					
Non-user	NA	NA	NA	NA	NA
1–5 times	0.12	–0.01	–0.08	–0.01	–0.01
6–10 times	0.27	–0.04	0.06	0.08	0.02
>10 times	–0.44	–0.15	–0.43	–0.24	–0.07
Alcohol frequency (per 15 days)					
Nonalcoholic	NA	NA	NA	NA	NA
1–3 times	–0.04	–0.02	0.03	0.03	–0.02
4–10 times	0.17	0.20	–0.19	–0.20	0.09
>10 times	0.02	0.30	0.29	0.25	0.34

5.1.1 Impact of age on functional parameter

First, we categorized the semen samples into six age groups to analyse the effect of age on semen characteristic. The groups were defined as ages below 25 years, between 26-30 years, 31-35 years, 36-40 years, 41-45 years, and above 45 years. The table 4 and figure 30 shows Semen Parameters for each group. As per the value mentioned in the table 4, we appear to conclude that the sperm count value is directly proportional to the age factor and found higher at age of >46yrs (37.05 M/ml). The total motility and progressive motility of sperm were observed to be highest (45.76%, 25.51%) in the age group of <25 years. The age factor does not affect the morphology of the semen samples. However, as age increases, semen volume

remains relatively stable (in between 25-45 yrs), while before and after this age it shows a slight decline.

Figure 30 a: Age vs sperm count

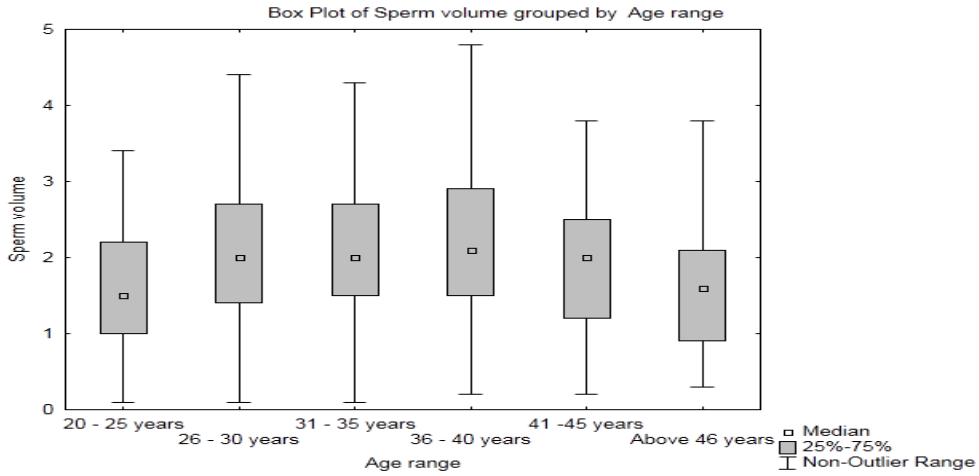


Figure 30 b: Age vs Semen volume

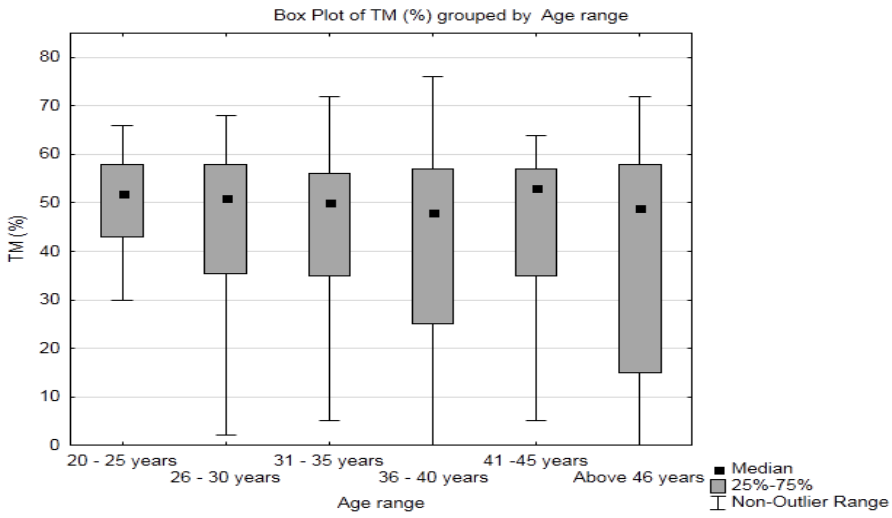


Figure 30 c: Age vs Total Motility

However, the age group of >45 years is a causative factor for higher combine effect values. These findings suggest that age appears to influence certain semen parameters, potentially impacting male fertility and reproductive health.

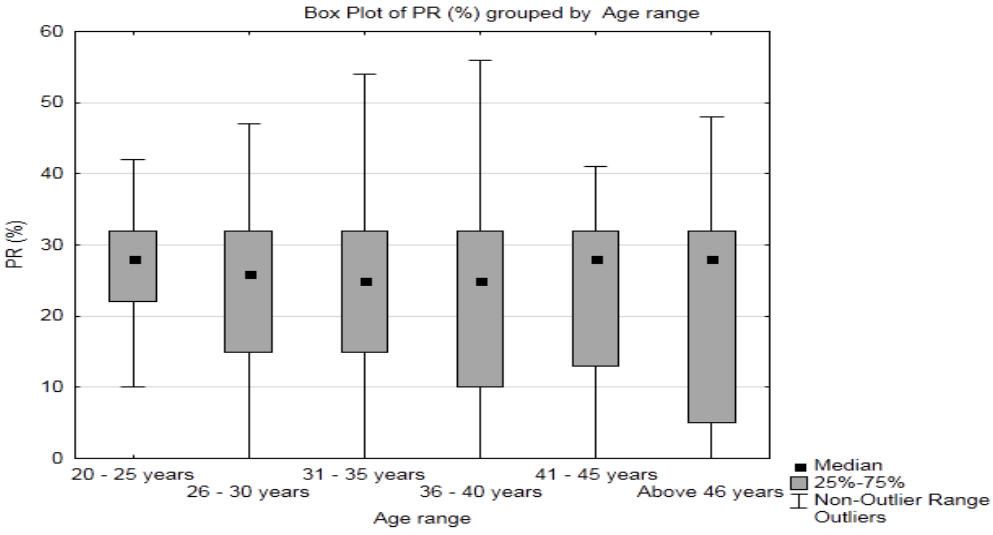


Figure 30 d: Age vs Progressive Motility

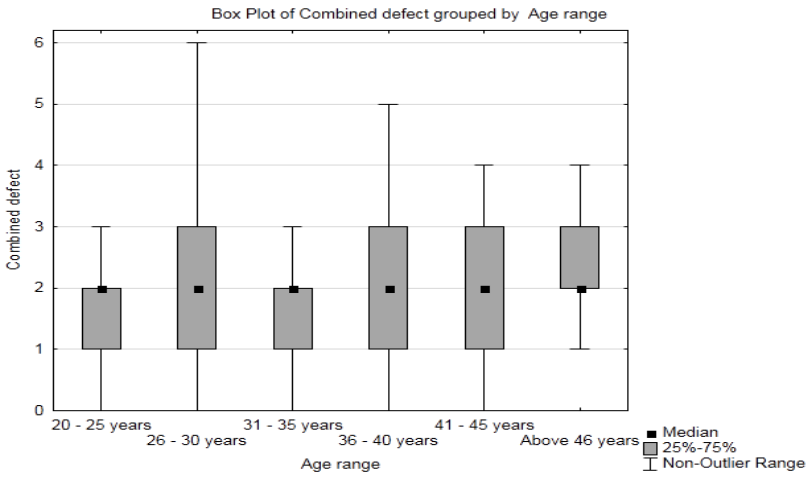


Figure 30 e: Age vs Combine defects

Figure 30: Age groups vs semen parameters, age range 20 to 55 years (a) represents the impact of age on sperm count, (b) represents the impact of age on semen volume, (c) represents the impact of age on total motility, (d) represents the impact of age on progressive motility, (e) represents the impact of age on morphology, (f) represents the impact of age on combine defects

Furthermore, p-values were evaluated for all groups, and correlation coefficients were calculated. From the table, it appears to be observed that the p-values range between 0.01 and 0.85, indicating that the few observed differences in semen parameters among the age groups are not statistically significant. However, correlation coefficients between -0.03 and 0.02 indicated the intensity and direction of the linear association between age and semen characteristics within each age group. 1 is ideal positive correlation, 0 represents no correlation, and -1 is ideal negative correlation. Close correlation coefficients imply a strong positive linear relationship among age and semen parameters across age groups. This suggests that as age increases within each age group, there is a tendency for semen parameters to increase as well. Hence, the p-values indicate that there is no significant alteration in few semen parameters between the age groups, while the correlation coefficient values suggest a strong positive connection among age and semen quality within each age group.

5.1.2 Impact of BMI

A BMI is categorised as underweight (<18.5 kg/m^2), normal (18.5–24.9 kg/m^2), overweight (25–29.9 kg/m^2) and obese (≥30 kg/m^2) to to assess the influence of obesity on spermatozoa.

According to the outcomes got in the above table 4, we appear to observe that sperm count, sperm motility and sperm morphology values declined in the group of underweight BMI and Obese BMI patients. Whereas Normal weight BMI group patients showed the highest average value of sperm count (32.73 M/ml), total motility (45.11 %), progressive motility (24.87 %) and Morphology (1.74%) as well. Volume of semen remains stables in all BMI groups except obese group. Declined value of semen volume was observed in obese group.

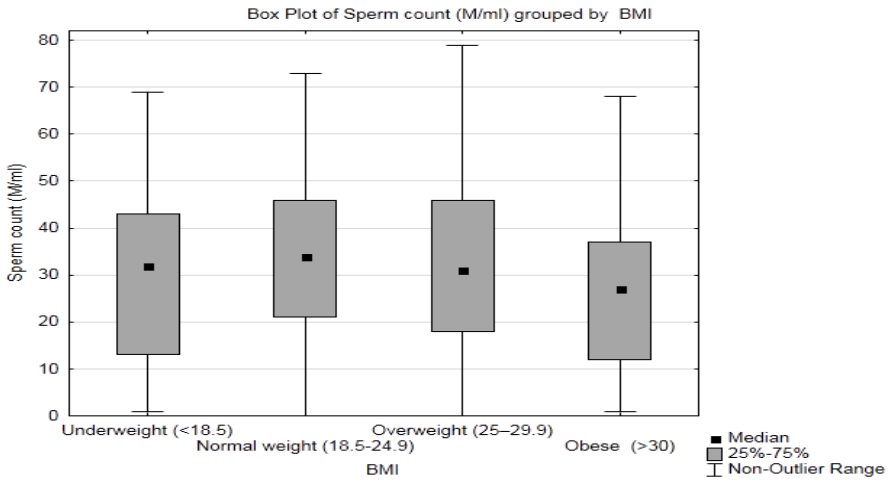


Figure 31a: Impact of BMI on Sperm count

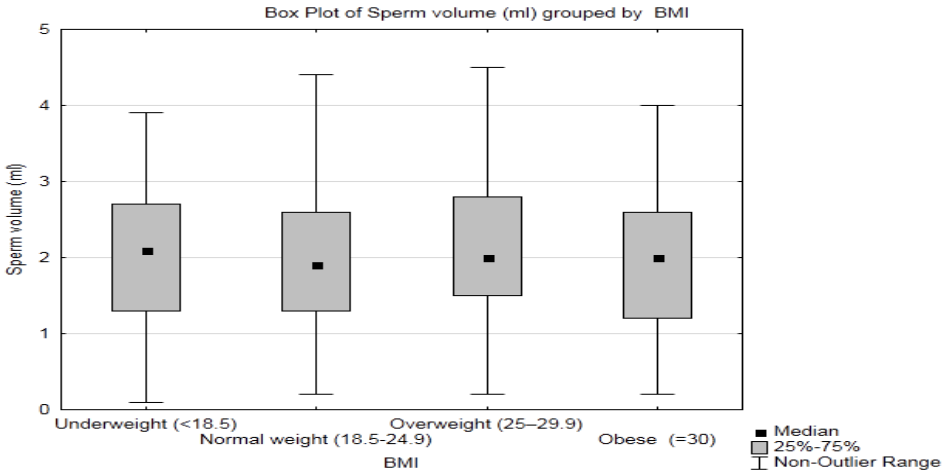


Figure 31b: Impact of BMI on Semen volume

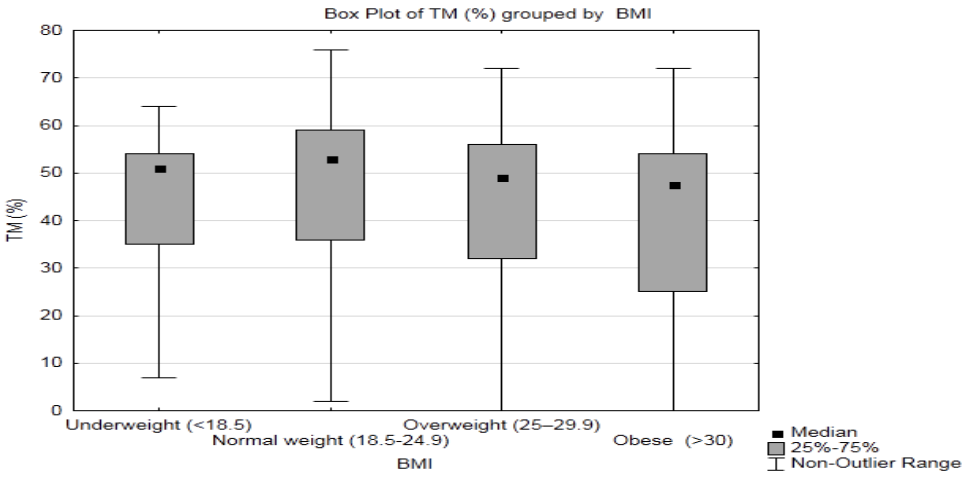


Figure 31c: Impact of BMI on Total motility

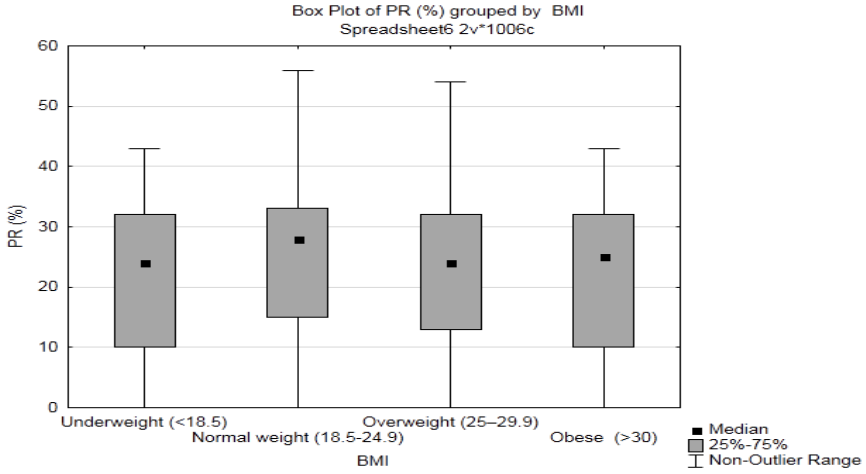


Figure 31d: Impact of BMI on Progressive Motility

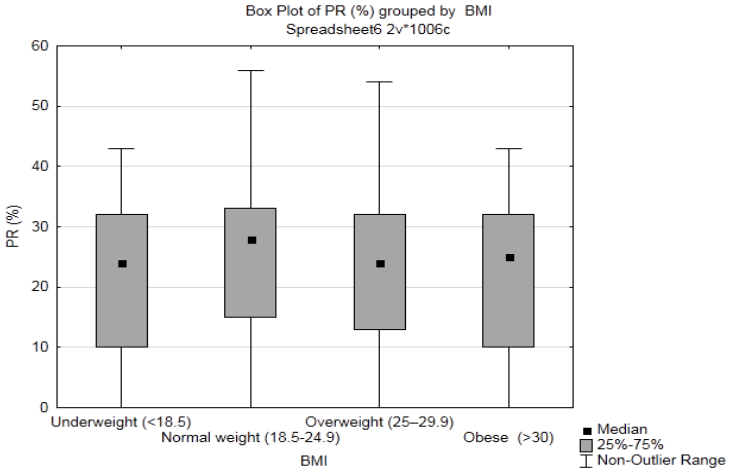


Figure 31e: Impact of BMI on Morphology

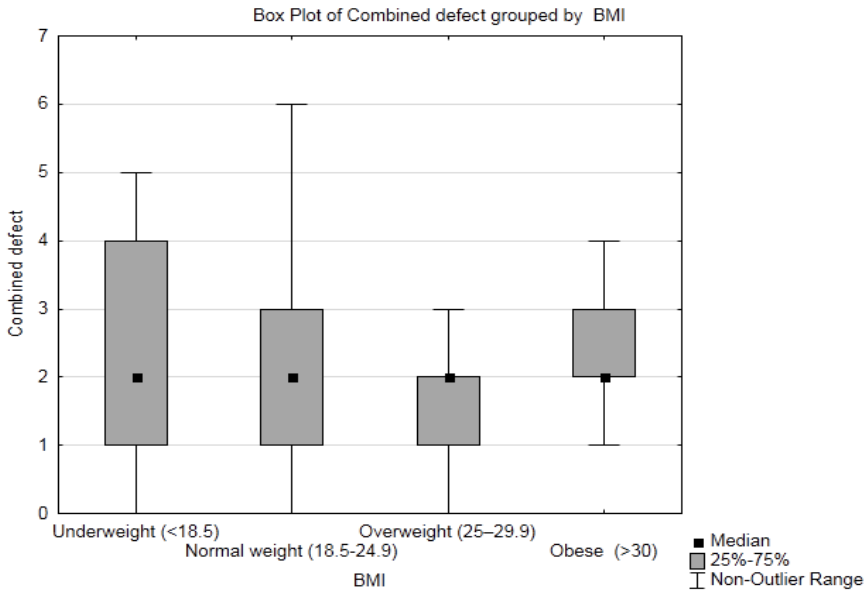


Figure 31f: Impact of BMI vs combined defects

Figure 31: BMI groups vs semen parameters, age range 20 to 55 years (a) represents the impact of BMI on sperm count, (b) represents the impact of BMI on semen volume, (c) represents the impact of BMI on total motility, (d) represents the impact of BMI on progressive motility, (e) represents the impact of BMI on morphology, (f) represents the impact of BMI on combine defects

All parameters are shown in figure 31. The p-values and correlation coefficients provided in the table offer insights into the statistical analysis shown to evaluate the connection between BMI and semen parameters. These values specifically pertain to the underweight, overweight, and obese groups in comparison to the normal weight group. The obtained p-values (0.00 to 0.06) indicate the statistical significance as of the observed disparities in semen parameters among the BMI groups. A p-value below 0.05 suggests statistically important differences in semen characteristics within the underweight and obese groups, indicating the influence of BMI.

Regarding the correlation coefficients (-0.09 to 0.07), their proximity to 1 signifies a strong positive linear relationship. These coefficients quantify the strength and direction of the linear relationship between BMI and semen parameters within each BMI group. Consequently, as BMI increases within each group, there is a tendency for semen parameters to increase as well. Thus, the p-values underscore the influence of BMI on semen quality, while the high

correlation coefficients suggest a robust positive linear relationship between BMI and the analysed semen parameters.

5.1.3 Impact of Lifestyle Factors on semen quality

5.1.3.1 Effect of Cigarette Smoking

To evaluate the impact of cigarette smoking, patients were divided into smoker and non-smoker groups, comprising 166 and 840 samples, respectively. The results indicate lower values of semen parameters among the smoker group. Decreased parameters like volume, sperm count, motility, and morphology suggest compromised semen quality, which appears to have implications for fertility and successful conception. As per following table 6, smoking significantly impact on sperm count, sperm motility and progressive motility

Table 6: Impact of Cigarette smoking on semen parameter

	Smoking Vs Volume			
	No. of participants	Average	Std. Deviation (±)	p value
Smoker	166	2.01	0.98	0.26
Non-smoker	840	2.12	1.19	

	Smoking Vs Sperm count			
	No. of participants	Average	Std. Deviation (±)	p value
Smoker	166	28.71	19.82	0.03
Non-smoker	840	32.02	17.52	

	Smoking Vs TM (%)			
	No. of participants	Average	Std. Deviation (±)	p value
Smoker	166	39.72	20.51	0.03
Non-smoker	840	43.23	19.11	

	Smoking Vs PR (%)			
	No. of participants	Average	Std. Deviation (±)	p value
Smoker	166	21.21	13.29	0.04
Non-smoker	840	23.39	12.59	

	Smoking Vs Morphology			
	No. of participants	Average	Std. Deviation (±)	p value
Smoker	166	1.48	1.02	0.15

Non-smoker	840	1.60	1.05	

	Smoking Vs combined defect			
	No. of participants	Average	Std. Deviation (±)	p value
Smoker	166	2.28	1.39	0.01
Non-smoker	840	2.01	1.22	

*The age range of subjects was 20 to 55 years

As per table 4, the samples were further separated into four groups depending on daily cigarette consumption as 0–5, 1–5, 6–10, and more than 10. The non-smokers had higher volume, sperm count, total and progressive motility, and morphology. However, an increasing number of cigarettes consumed shows a cumulative negative impact on semen parameters, with a corresponding increase in combined defects.

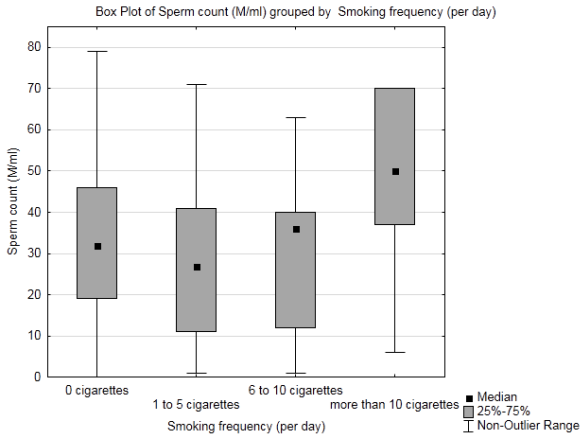


Figure 32 a: Smoking vs sperm count

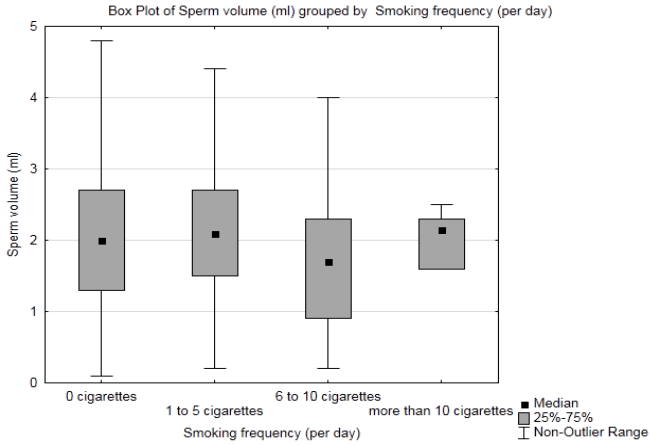


Figure 32 b: Smoking vs semen volume

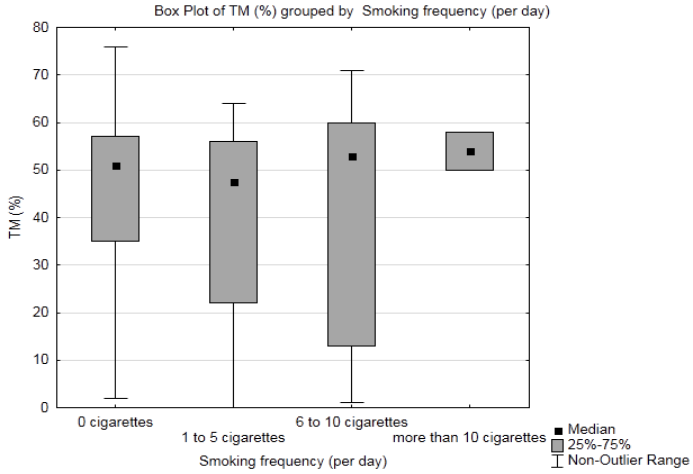


Figure 32c: Smoking vs Total Motility

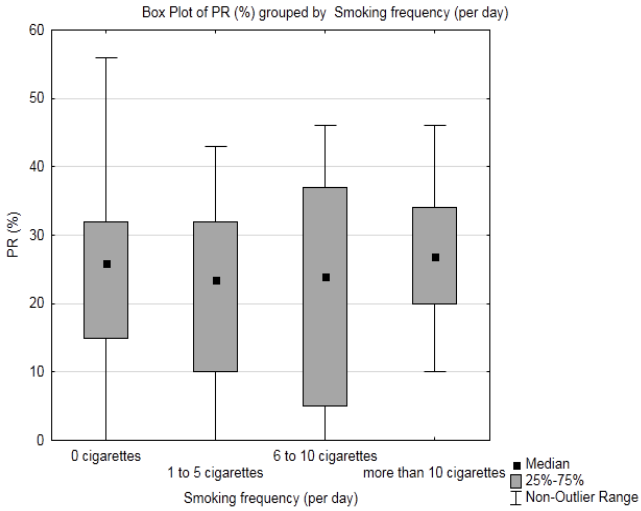


Figure 32d: Smoking vs Progressive Motility

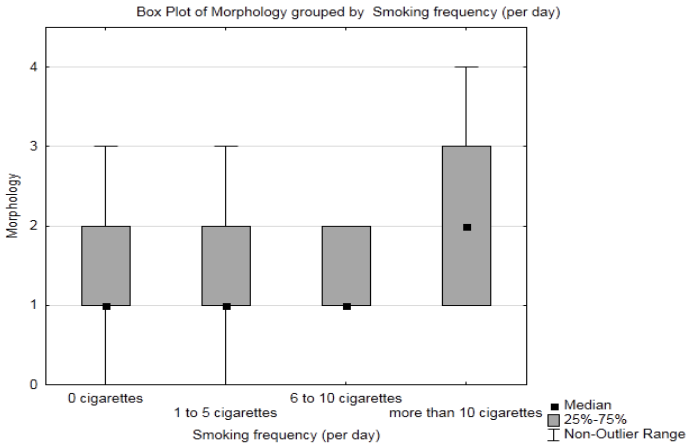


Figure 32e: Smoking vs Morphology

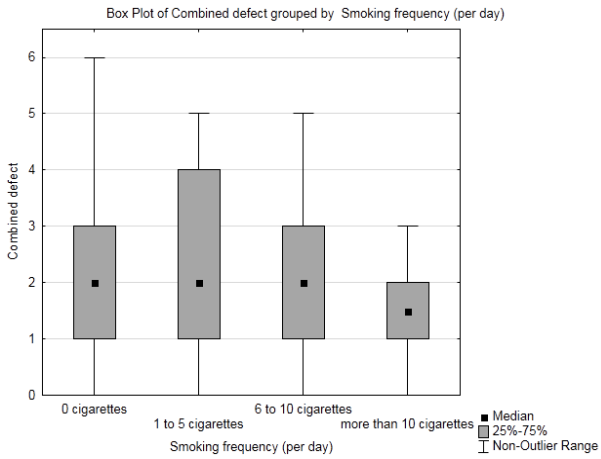


Figure 32f: Smoking vs combined defect

Figure 32: Cigarette smoking frequency vs semen parameters, age range 20 to 55 years (a) represents the impact of cigarette smoking on sperm count, (b) represents the impact of cigarette smoking on semen volume, (c) represents the impact of cigarette smoking on total motility, (d) represents the impact of cigarette smoking on progressive motility, (e) represents the impact of cigarette smoking on morphology, (f) represents the impact of cigarette smoking on combine defects

As per figure 32 there was a significant impact of smoking on semen parameters. Cigarette smoking affects semen parameters with a p-value below 0.05. The correlation coefficient shows a strong positive linear association between smoking and semen parameters. This shows a tight link between cigarette smoking and semen parameter alterations. The substantial tendency for semen parameters to rise with cigarette smoking supports the argument that smoking may harm semen quality or parameters. These findings demonstrate that smoking negatively impacts semen quality, underlining the need of quitting smoking to improve semen quality. The p-value was evaluated for each group, comparing individuals who smoke 1-5 cigarettes, 6-10 cigarettes, and more than 10 cigarettes per day against the no-smoking group. The obtained p-values were 0.01, 0.04, and 0.05, all of which are lower than the threshold of 0.05. This indicates that the semen parameters are statistically affected by cigarette smoking. Furthermore, the correlation coefficient suggests a strong positive linear relationship between cigarette smoking and the analysed semen parameters. This high correlation coefficient indicates a consistent and direct association between the frequency of cigarette smoking and the observed changes in the parameters.

5.1.3.2 Impact of Tobacco Consumption

Tobacco consumption is analysed as a risk factor affecting semen quality, comparing groups of tobacco-consuming participants (236 samples) and non-tobacco-consuming participants (770 samples) as per table 7. According to the results, direct relationship was seen between tobacco consumption and all semen functional parameters.

Table 7: Impact of Tobacco consumption on semen parameter

Tobacco use Vs Volume				
	No. of participants	Average	Std. Deviation (±)	p value
Yes	242	2.02	1.11	0.20
No	764	2.12	1.17	

Tobacco use Vs Sperm count				
	No. of participants	Average	Std. Deviation (±)	p value
Yes	242	33.24	18.60	0.08
No	764	30.91	17.72	

Tobacco use Vs TM (%)				
	No. of participants	Average	Std. Deviation (±)	p value
Yes	242	44.36	19.54	0.11
No	764	42.11	19.31	

Tobacco use Vs PR (%)				
	No. of participants	Average	Std. Deviation (±)	p value
Yes	242	24.03	12.76	0.16
No	764	22.72	12.71	

Tobacco use Vs Morphology				
	No. of participants	Average	Std. Deviation (±)	p value
Yes	242	1.64	1.03	0.37
No	764	1.57	1.06	

Tobacco use Vs combined defect				
	No. of participants	Average	Std. Deviation (±)	p value
Yes	242	2.14	1.34	0.20
No	764	2.03	1.22	

*The age range of subjects was 20 to 55 years

When observing semen quality in groups consuming tobacco based on daily consumption, it is evident that as the quantity of cigarettes smoked per day upsurges, there was a regular decrease in semen volume, sperm count, and total motility. Additionally, progressive motility and morphology show a decline with an increasing number of cigarettes smoked per day. However, in case of tobacco these results indicate a negative influence on sperm quality, leading to the partial conclusion that tobacco consumption is inversely proportional to sperm parameters. Those who are consume tobacco less than 1-5 times per day showing positive impact on semen parameter. The p-value of >0.05 suggests a statistically non-significant difference in semen quality between the groups with tobacco consumption (N=242) and without tobacco consumption (N=764). This indicates that the observed difference in semen quality is unlikely to occur due to random chance alone. However, it is important to note that the p-value is close to the significance as threshold of 0.05. While it does suggest a significant difference, further investigation is needed to determine the magnitude of the difference and confirm its significance. On the other hand, the correlation coefficient of (-0.02, -0.03, -0.04) indicates a very strong negative linear relationship between tobacco consumption and the analysed semen parameters.

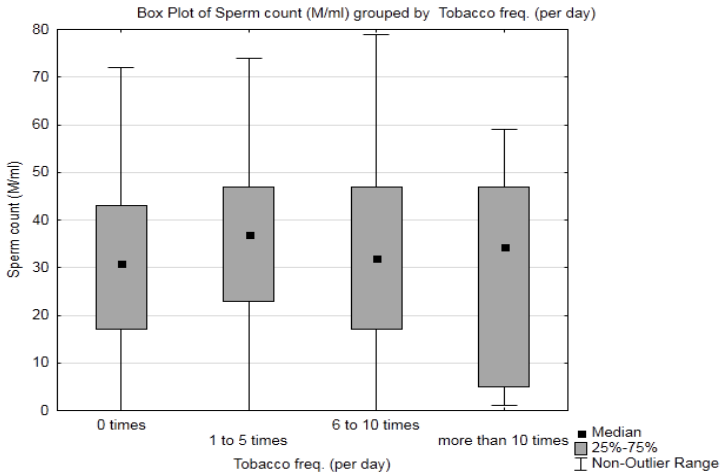


Figure 33a: Tobacco Consumption Vs Sperm Count

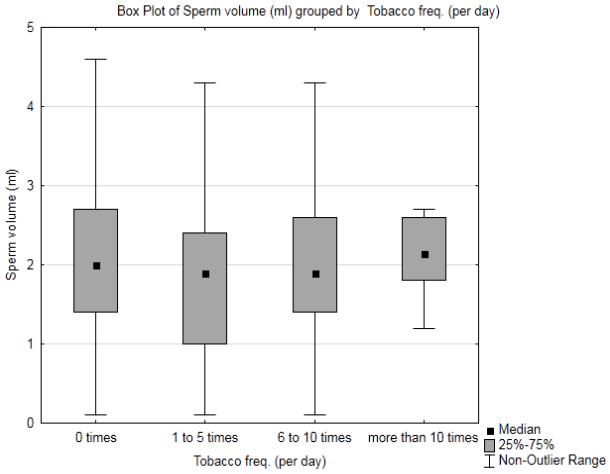


Figure 33b: Tobacco Consumption Vs Semen Volume

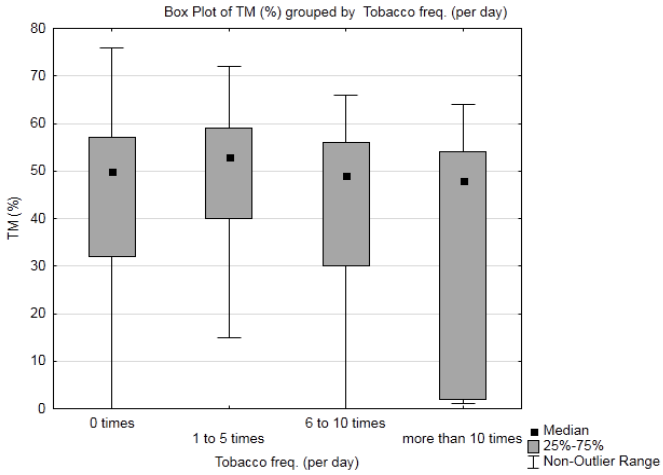


Figure 33c: Tobacco Consumption Vs Total Motility

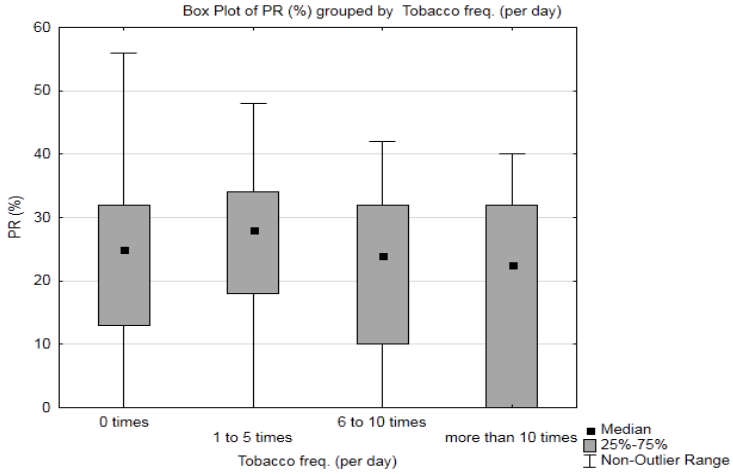


Figure 33d: Tobacco Consumption Vs Progressive Motility

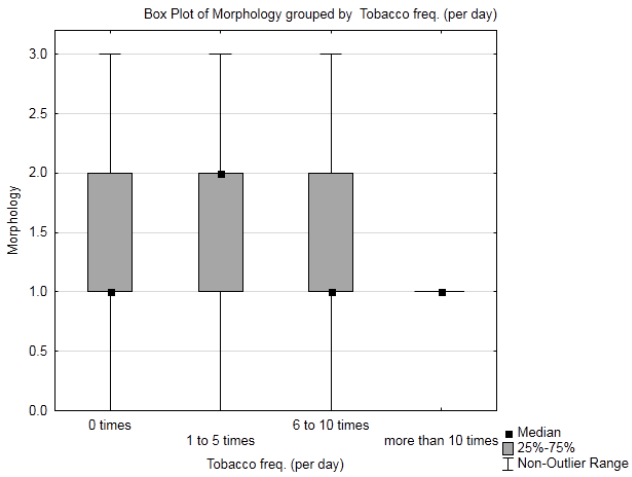


Figure 33e: Tobacco Consumption Vs Morphology

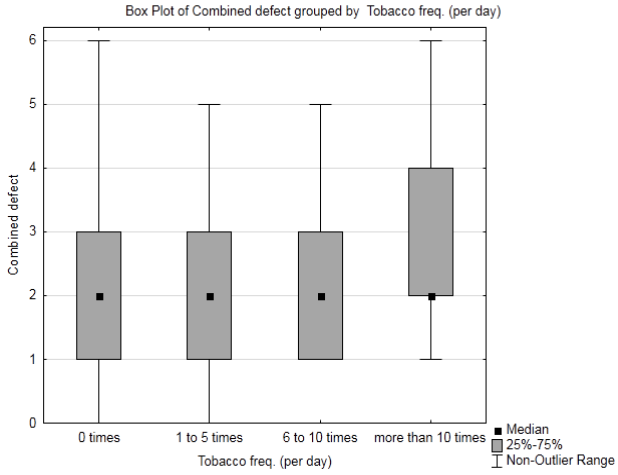


Figure 33f: Tobacco Consumption Vs Combined Defect

Figure 33: Tobacco consumption vs semen parameters, age range 20 to 55 years (a) represents the impact of tobacco consumption on sperm count, (b) represents the impact of tobacco consumption on semen volume, (c) represents the impact of tobacco consumption on total motility, (d) represents the impact of tobacco consumption on progressive motility, (e) represents the impact of tobacco consumption on morphology, (f) represents the impact of tobacco consumption on combine defects

This proposes a highly consistent and negative association between tobacco consumption and changes in semen quality within the respective groups. The high correlation coefficient implies that as tobacco consumption increases, there is a strong

tendency for semen quality to be affected as well (see figure 33). The impact of tobacco consumption on semen parameters becomes evident when comparing different levels of tobacco consumption (see table 4). The adverse impact of tobacco consumption on semen parameters suggests the importance of reducing or quitting tobacco use for better reproductive health.

When comparing different groups based on the quantity of tobacco consumption per day against the no tobacco consumption group, distinct p-values are observed. The p-values for the total motility, progressive motility and morphology are 0.04, 0.01 and 0.00, respectively. These values suggest a significant impression of tobacco consumption on semen quality. However, in the case of sperm count, semen volume and combined defect p-value is more than 0.05, representing a absent of statistical significance as in the observed differences. Furthermore, the correlation coefficients of -0.02, -0.02, -0.03 and -0.04 for the parameter of Semen volume, total motility, progressive motility, and morphology, respectively, demonstrate a strong negative relationship between tobacco consumption and the analysed semen parameters. Whereas, the correlation coefficients of 0.02 and 0.09, for the parameter of sperm count and combined defect, respectively. These high correlation coefficients signify a consistent and direct association between the quantity of tobacco consumed and the observed changes in semen parameters.

Hence, the obtained p-values suggest a significant effect of tobacco consumption on semen parameters, while the lack of statistical significance as in few parameters indicate a possible threshold effect. Additionally, the high correlation coefficients indicate a strong positive linear relationship between tobacco consumption quantity and the observed changes in semen parameters across all groups.

5.1.3.3 Impact of Alcohol consumption

Furthermore, alcohol consumption was evaluated for its impact on semen parameters. Participants are separated into two groups: alcohol-consuming and non-consuming categories figure 34. The following table 8, displays higher values of sperm count and sperm morphology in the alcohol non-consumption group but it was nonsignificant. Therefore, it was decided that there was no correlation between alcohol consumption

and semen quality. The p-value being above the conventional significance as threshold of 0.05 suggests that the observed variances in semen parameters between the alcohol-consuming and non-consuming groups may not be statistically significant.

Table 8: Impact of alcohol consumption on semen parameter

	Alcohol intake Vs Volume			
	No. of participants	Average	Std. Deviation (±)	p value
Yes	380	2.05	1.15	0.27
No	626	2.13	1.16	
	Alcohol intake Vs Sperm count			
	No. of participants	Average	Std. Deviation (±)	p value
Yes	379	31.65	19.18	0.81
No	625	31.37	17.20	
	Alcohol intake Vs TM (%)			
	No. of participants	Average	Std. Deviation (±)	p value
Yes	379	42.97	19.43	0.67
No	625	42.43	19.39	
	Alcohol intake Vs PR (%)			
	No. of participants	Average	Std. Deviation (±)	p value
Yes	379	23.35	12.71	0.52
No	625	22.82	12.76	
	Alcohol intake Vs Morphology			
	No. of participants	Average	Std. Deviation (±)	p value
Yes	379	1.56	1.00	0.61
No	625	1.60	1.08	
	Alcohol intake Vs combined defect			
	No. of participants	Average	Std. Deviation (±)	p value
Yes	379	2.11	1.27	0.29
No	625	2.02	1.25	

*The age range of subjects was 20 to 55 years

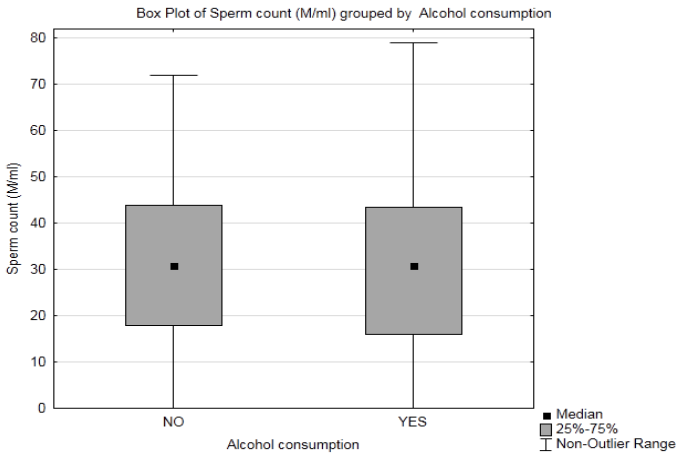


Figure 34 a. Alcohol Consumption Vs Sperm Count

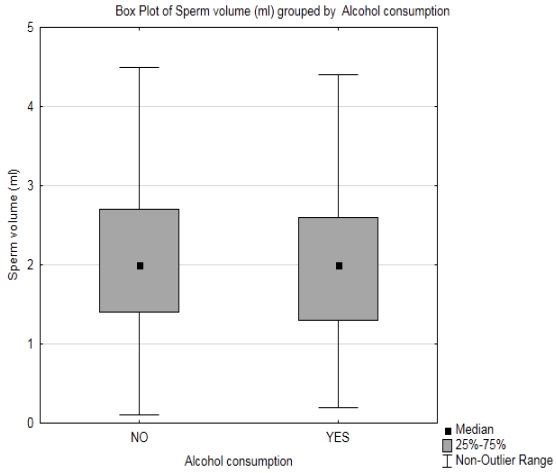


Figure 34 b. Alcohol Consumption Vs Semen Volume

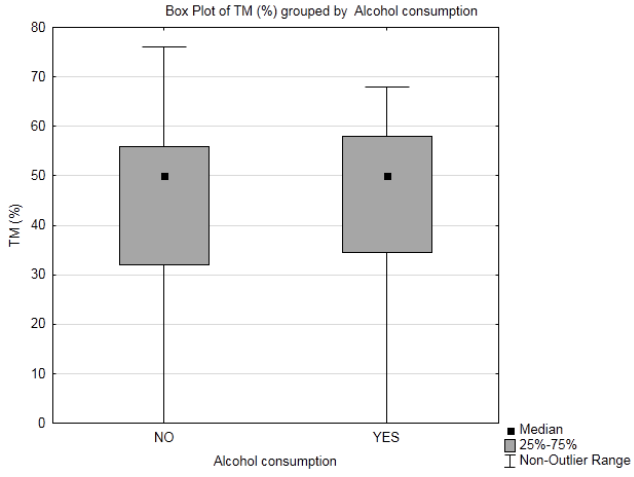


Figure 34 c. Alcohol Consumption Vs Total Motility

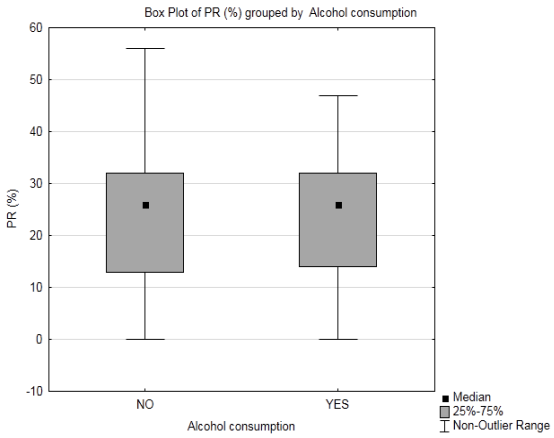


Figure 34 d. Alcohol Consumption Vs Progressive Motility

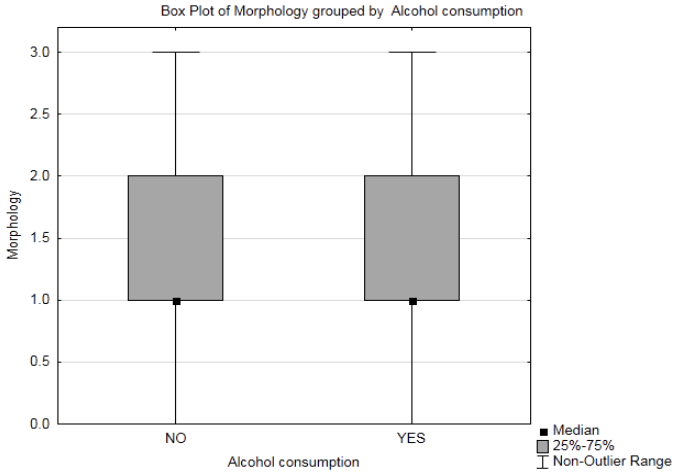


Figure 34 e. Alcohol Consumption Vs Morphology

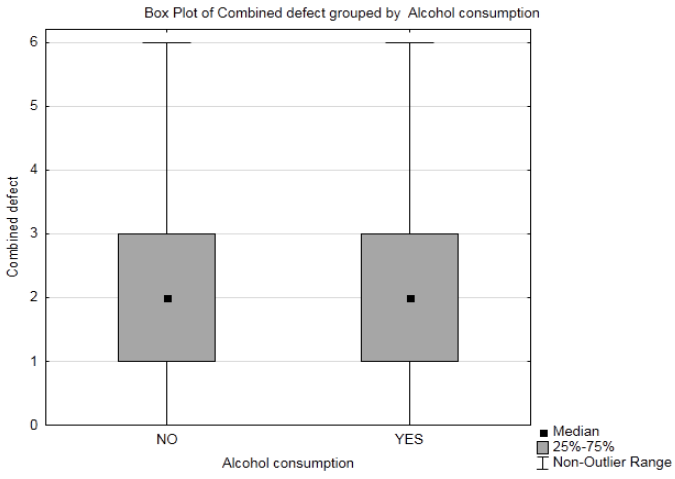


Figure 34 f. Alcohol Consumption Vs Combined Defect

Figure 34: Alcohol consumption vs individual parameters, age range 20 to 55 years (a) represents the impact of alcohol consumption on sperm count, (b) represents the impact of alcohol consumption on semen volume, (c) represents the impact of alcohol consumption on total motility, (d) represents the impact of alcohol consumption on progressive motility, (e) represents the impact of alcohol consumption on morphology, (f) represents the impact of alcohol consumption on combine defects.

5.1.4 Impact of Diet on semen characteristic

In the table 9 below, the impact of a veg and non-veg diet on semen parameters were observed. The veg group, consisting of 188 samples, exhibits higher sperm count, TM (total motility), PR (progressive motility), and morphology compared to the non-veg group with 818 samples. However, the non-veg group shows a slight increase in volume. Additionally, the combined defect's is higher in the non-veg diet group.

These outcomes advise that following a veg diet may have a positive impression on semen quality compared to a non-veg diet. The veg group demonstrates better semen quality in sense of sperm count, motility, progressive motility, and morphology.

Table 9: Impact of veg and non-veg diet on semen parameters

	Food habit Vs Volume			
	No. of participants	Average	Std. Deviation (±)	p value
Non-vegetarian	818	2.12	1.17	0.24
Vegetarian	188	2.01	1.08	
	Food habit Vs Sperm count			
	No. of participants	Average	Std. Deviation (±)	p value
Non-vegetarian	818	30.89	18.07	0.03
Vegetarian	188	33.98	17.23	
	Food habit Vs TM (%)			
	No. of participants	Average	Std. Deviation (±)	p value
Non-vegetarian	818	42.39	19.67	0.38
Vegetarian	188	43.77	18.10	
	Food habit Vs PR (%)			
	No. of participants	Average	Std. Deviation (±)	p value
Non-vegetarian	818	22.86	12.86	0.38

Vegetarian	188	23.77	12.13	

	Food habit Vs Morphology			
	No. of participants	Average	Std. Deviation (±)	p value
Non-vegetarian	818	1.55	1.04	0.06
Vegetarian	188	1.71	1.08	

	Food habit Vs combined defect			
	No. of participants	Average	Std. Deviation (±)	p value
Non-vegetarian	818	2.08	1.28	0.19
Vegetarian	188	1.95	1.12	

*The age range of subjects was 20 to 55 years

The p-value of 0.03 for sperm count shown that there is a statistically significant effect of the veg diet, suggesting potential differences between the two groups. For other parameters average value shows higher but p-value observed more than 0.05, This suggests that the veg or non-veg diet may have a consistent and direct association with the observed changes in semen parameters. However, a more comprehensive understanding of the relationship between diet type and semen parameters appears to be studied further.

5.1.5 Correlation of Medical History with semen characteristics

Semen parameters analysed for patients with medical does not showing any impact as compared to individuals with no medical history. However, as per table 10, the differences in other semen parameters are relatively small. Thus, according to the results, we have not observed any impact of previous medical history on semen parameters.

Table 10: Impact of medical history on semen parameter

	Presence of medical history Vs Volume			
	No. of participants	Average	Std. Deviation (±)	p value
Yes	251	2.03	1.01	0.28
No	755	2.12	1.20	

	Presence of medical history Vs Sperm count			
	No. of participants	Average	Std. Deviation (±)	p value

Yes	251	33.24	19.37	0.07
No	755	30.88	17.43	

Presence of medical history Vs TM (%)				
	No. of participants	Average	Std. Deviation (±)	p value
Yes	251	42.34	18.80	0.77
No	755	42.75	19.58	

Presence of medical history Vs PR (%)				
	No. of participants	Average	Std. Deviation (±)	p value
Yes	251	23.09	11.99	0.93
No	755	23.01	12.97	

Presence of medical history Vs Morphology				
	No. of participants	Average	Std. Deviation (±)	p value
Yes	251	1.61	1.09	0.65
No	755	1.57	1.04	

Presence of medical history Vs combined defect				
	No. of participants	Average	Std. Deviation (±)	p value
Yes	251	2.04	1.23	0.78
No	755	2.06	1.26	

*The age range of subjects was 20 to 55 years

As per results there was no significant difference in mean value between the two parameters. Evaluating the p-value and correlation coefficient, we appear to observe the relationship between the two groups in terms of medical history and semen parameters. The whole p-values of >0.05 suggests that there is no significant impact of medical history on semen parameters.

5.1.6 Use of Filtered Water and Non-filtered Water associated with semen parameter

Water is an important dietary factor analysed for its impact on semen quality. The participants have been alienated as per table 11, into two groups based on their use of water purifiers. The results obtained, as shown in the table 11, include 389 participants

in the group consuming non-filtered water and 617 participants using water purifiers for filtered water.

Table 11: Impact of the use of filter water and non-filter water on semen parameter

	Availability of RO water Vs Volume			
	No. of participants	Average	Std. Deviation (±)	p value
Available	617	2.14	1.19	0.17
Not-available	389	2.03	1.10	
	Availability of RO water Vs Sperm count			
	No. of participants	Average	Std. Deviation (±)	p value
Available	617	31.98	17.19	0.26
Not-available	389	30.67	19.10	
	Availability of RO water Vs TM (%)			
	No. of participants	Average	Std. Deviation (±)	p value
Available	617	43.93	19.00	0.01
Not-available	389	40.62	19.84	
	Availability of RO water Vs PR (%)			
	No. of participants	Average	Std. Deviation (±)	p value
Available	617	23.64	12.33	0.06
Not-available	389	22.07	13.30	
	Availability of RO water Vs Morphology			
	No. of participants	Average	Std. Deviation (±)	p value
Available	617	1.56	1.02	0.35
Not-available	389	1.62	1.09	
	Availability of RO water Vs combined defect			
	No. of participants	Average	Std. Deviation (±)	p value
Available	617	1.97	1.15	0.01
Not-available	389	2.19	1.40	

*The age range of subjects was 20 to 55 years

Based on the values obtained from the analysis, it is evident that the subjects who drink water from water purifiers have higher values for semen volume, sperm count, total motility, and progressive motility. Therefore, it appears to be observed that filtered water has a significant influence on semen parameters. Also, the p-value for the parameter of Motility is 0.01 suggests that there is an impact of filtered water on semen parameters compared to the group consuming non-filtered water, it does reach statistical significance. For other parameters p-values observe >0.05, This indicates a potential but not conclusive effect of filtered water on semen parameters.

5.1.7 Impact of Fast Food on semen quality

The impact of consuming fast food on semen quality was examined in comparison to a group that does not consume fast food. The group that does not consume fast food (811 samples) exhibited higher values in semen aspects like volume, sperm count, total motility (TM), progressive motility (PR), and morphology, along with a reduced combined defect has shown in table 12. In contrast, the group consuming fast food including 195 samples showed lower semen parameters and an increased combined defect.

Table 12: Impact of fast food on semen parameter

Fast Food	Volume (ml)	Sperm count (M/ml)	TM (%)	PR (%)	Morpho (%)	Combine defect	P-value	Correl Coeff.
(NO) (N=811)	2.14 ±1.16	32.05± 17.77	43.14± 19.18	23.29± 12.67	1.63± 1.09	2.02± 1.27	0.0392	0.9997
(YES) (N=195)	1.93 ±1.12	29.07± 18.55	40.61± 20.12	21.95± 12.97	1.38± 0.81	2.19 ±1.18		

*The age range of subjects was 20 to 55 years

These results recommend that consuming fast food may have a detrimental effect on semen parameters, resulting in lower values for volume, sperm count, TM, PR, and morphology, as well as an increased combined defect. The observed differences in

semen parameters amongst the two groups indicate a potential impact of fast-food consumption on male reproductive health. Furthermore, the p-value of 0.0392 among the groups consuming and not consuming fast food suggests a statistically significant change in semen parameters. This indicates that the observed disparities in semen parameters between the two groups are unlikely to occur by chance alone.

Moreover, the correlation coefficient of 0.9997 proposes a strong positive linear association amid fast food consumption and the analysed semen parameters. This implies that there is a consistent and direct association between fast food consumption and the observed changes in semen parameters. Therefore, consuming fast food have a negative association with semen parameters, as indicated by the lower values in various parameters and the increased combined defect. These results highlight the importance of keeping a healthy diet to support optimal male reproductive health.

5.1.8 Food Supplement and their effect on semen

The influence of food supplement consumption on semen parameters was observed in two groups as per table 13, including a group of 82 participants those consuming food supplements and a group of 924 participants those not consuming food supplements. The results showed that in the food supplement non-consuming group, different aspects of semen such as volume, sperm count, total motility, progressive motility, and morphology were higher compared to the food supplement consuming group. However, the combine defect was slightly higher in the food supplement consuming group.

Table 13: Impact of a food supplement on semen parameter

Food supplement	Volume (ml)	Sperm count (M/ml)	TM (%)	PR (%)	Morpho (%)	Combine defect	P-value	Correl Coeff.
(NO) (N=924)	2.10± 1.14	31.72±17.94	42.84± 19.38	23.23± 12.75	1.60± 1.06	2.03± 1.24	0.0413	0.9993
(YES) (N=82)	2.05± 1.28	28.71±17.92	40.56± 19.45	20.79± 12.31	1.43± 0.89	2.30± 1.36		

*The age range of subjects was 20 to 55 years

These results specify that the consumption of food supplements may have an impact on semen parameters, as reflected by the lower values observed in volume, sperm count, TM, PR, and morphology in the consuming subjects compared with the non-consuming subjects. To some extent higher combine defect in the consuming group suggests potential irregularities in semen quality. Additionally, the p-value is calculated for the groups consuming food supplements compared to the groups not consuming them. The obtained p-value of 0.0413 suggests a statistically significant alteration in semen parameters amongst the two sets of subjects.

Furthermore, the correlation coefficient of 0.9993 specifies a strong positive linear relationship among the consumption of food supplements and the analysed semen parameters. This implies a consistent and direct link between the use of food supplements and the observed changes in semen parameters.

Hence, the consumption of food supplements has an impact on semen parameters, as evidenced by the lower values observed in various semen parameters in the group consuming food supplements related to the non-consuming group. The results above suggest food supplements may affect male reproductive health. However, further investigate is required to know the specific mechanisms and possible pros and cons of food supplement consumption on semen parameters.

5.1.9 Impact of Tea/coffee (caffeine intake) consumption on semen parameters

An impact of tea or coffee consumption on semen parameters were observed in two groups: one consisting of individuals consuming tea/coffee (858 participants) and the other not consuming tea/coffee (148 participants). The results in table 14, show significant semen parameter disparities between categories.

Table 14: Impact of tea/coffee on semen parameter

	Tea/coffee intake Vs Volume			
	No. of participants	Average	Std. Deviation (±)	p value
Yes	858	2.13	1.19	0.04
No	148	1.91	0.93	

Caffeine intake Vs Sperm count				
	No. of participants	Average	Std. Deviation (±)	p value
Yes	858	32.17	17.89	0.00
No	148	27.41	17.80	

Caffeine intake Vs TM (%)				
	No. of participants	Average	Std. Deviation (±)	p value
Yes	858	43.23	19.20	0.02
No	148	39.27	20.17	

Caffeine intake Vs PR (%)				
	No. of participants	Average	Std. Deviation (±)	p value
Yes	858	23.57	12.70	0.00
No	148	19.95	12.52	

Caffeine intake Vs Morphology				
	No. of participants	Average	Std. Deviation (±)	p value
Yes	858	1.58	1.02	0.98
No	148	1.58	1.21	

Caffeine intake Vs combined defect				
	No. of participants	Average	Std. Deviation (±)	p value
Yes	858	2.02	1.20	0.03
No	148	2.26	1.50	

*The age range of subjects was 20 to 55 years

Specifically, parameters that includes semen volume, sperm count, total motility, and progressive motility show slight increases in the tea/coffee-consuming group, while comparing the comparable between the two groups. Similarly, there are no significant variations in sperm morphology. We appear to conclude from the data that tea/coffee consumption has a significant impact on semen parameters.

The p-values of sperm count, semen volume, total motility, progressive motility and combine defects are 0.00, 0.04, 0.02, 0.00 and 0.02 respectively, observed between the groups consuming and not consuming tea or coffee suggests a potential impact on

semen parameters. This suggests that these parameters differ across subgroups. Furthermore, the correlation coefficient of near 1 indicates a strong positive linear relationship. This implies a consistent link amongst tea/coffee consumption and semen parameters. However, the specific direction of this relationship showing whether consumption increases or decreases semen parameters, appears to not be determined only based on the correlation coefficient. Since semen parameters are increased in the consumption group compared to the non-consumption group, it suggests a positive impact. Therefore, while the p-value suggests an impact and the correlation coefficient indicates a consistent association, Further investigation is needed to determine how tea/coffee affects semen parameters and whether it leads to an increase or decrease in these parameters.

Table 15: Impact of tea/coffee groups on semen parameter

Tea/coffee per day	**Volume (ml)**	**Sperm count (M/ml)**	**TM (%)**	**PR (%)**	**Morpho (%)**	**Combine defect**	**P-value**	**Correl Coeff.**
0 (N=148)	2.09±1.14	31.46±18.03	42.61±19.41	22.99±12.76	1.59±1.05	2.05±1.25		
1-2 (N=357)	2.20±1.25	31.01±17.67	42.23±19.51	22.91±12.81	1.56±0.97	2.05±1.21	0.0956	1.0000
3-4 (N=353)	2.05±1.18	32.74±17.43	44.48±18.52	24.08±12.11	1.61±1.04	2.00±1.18	0.0475	1.0000
>5 (N=146)	2.14±1.04	31.22±19.53	43.62±20.02	23.94±13.77	1.57±1.09	1.99±1.25	0.1324	0.9997

*The age range of subjects was 20 to 55 years

Considering the increase in tea/coffee intake compared to the non-consuming group, we observed changes in semen parameters (see table 15). The group consuming 1-2 cups of coffee per day showed a higher volume of semen, while the group consuming 3-4 cups of tea/coffee per day exhibited higher values for sperm count, total motility

(TM), progressive motility (PR), and morphology. However, when tea/coffee consumption exceeded 5 cups per day, there was a decline in performance across semen parameters. No significant effect of tea/coffee on parameters associated with semen was found.

Furthermore, when comparing the impact of tea/coffee intake within each group to the non-consuming group, we obtained different p-values. These p-values provide statistical insights into the significance as of the observed variances between the set. The p-value of 0.0956 and correlation coefficient of 1.0000, when compared with the non-tea/coffee consuming group, suggest an impact on semen parameters for the group consuming 1-2 cups of tea/coffee per day, which consists of 357 participants. Similarly, the p-value of 0.0475 for the group consuming 3-4 cups of tea/coffee per day (353 participants) also indicates a potential impact on semen parameters. However, the p-value of 0.1324 for the group consuming more than 5 cups of tea/coffee per day (146 participants) suggests no statistically significant impact on semen parameters.

Furthermore, the correlation coefficients of 1.0000, 1.0000, and 0.9997 indicate a strong positive linear relationship between tea/coffee intake and semen parameters. This suggests a consistent association between the consumption of tea/coffee and fluctuations in semen parameters. In conclusion, tea/coffee intake may affect semen parameters depending on the parameter and consumption level. More research is needed to understand how tea/coffee affects semen parameters.

5.1.10 Fizzy Drink consumption and semen parameters

Consumption of carbonated drinks is investigated for its impact on semen parameters. The group that consumed cold drinks showed in table 16, a considerable deterioration in semen parameters compared to the non-consuming group. In contrast, the non-consuming group, consisting of 913 individuals, exhibited considerably higher values for volume, sperm count, total motility (TM), progressive motility (PR), and morphology of semen parameters. So, cold drinks may harm semen parameters.

Table 16: Impact of a fizzy drink on semen parameter

Cold Drink	Volume (ml)	Sperm count (M/ml)	TM (%)	PR (%)	Morpho (%)	Combine defect	P-value	Correl Coeff.
(NO) (N=913)	2.14± 1.17	31.87± 17.90	43.00± 19.22	23.29±12.73	1.61± 1.06	2.03±1.24	0.0318	0.9993
(YES) (N=93)	1.66± 0.93	27.59± 18.05	39.26± 20.76	20.49±12.52	1.33± 0.90	2.26 ±1.34		

*The age range of subjects was 20 to 55 years

The calculated p-value of 0.0318 indicates a relationship between the group consuming cold drinks and the non-consuming group. This implies a statistically significant variation in semen parameters across groups, further emphasizing the impact of carbonated drink consumption. Additionally, the correlation coefficient of 0.9993 proposes a strong relationship between cold drink consumption and semen parameters. This coefficient implies that there is a consistent association between the two variables, indicating that the consumption of cold drinks influences semen parameters. In summary, the observed data indicates that the consumption of Semen parameters drop with cold drinks. The statistical analysis supports this finding, with a significant-value and a strong correlation coefficient further reinforcing the relationship between cold drink consumption and semen parameters.

5.1.10.1 Combine effect of Caffeine intake (tea/coffee and Cold Drink) on semen quality

Caffeine intake, including tea/coffee and a soft drink, was evaluated for its combined impact on semen quality analysis. Participants were categorized into two groups: those consuming both tea/coffee and cold drink, and those not consuming either of them (see table 17). The results indicate that the caffeine-consuming group (87 participants) exhibited higher sperm count, total motility (TM), and progressive motility (PR), while the caffeine-non-consuming group (142 participants) showed slightly higher volume and morphology of semen. However, the combined effect of tea/coffee consumption on semen parameters was not found to be statistically significant.

Table 17: Impact of caffeine intake on semen parameter

Tea/coffee and Cold Drink	Volume (ml)	Sperm count (M/ml)	TM (%)	PR (%)	Morpho (%)	Combine defect	P-value	Correl Coeff.
(NO) (N=142)	1.96 ±0.92	28.27±17.62	40.21± 19.50	20.39±12.29	1.61± 1.23	2.18± 1.46	0.1226	0.9999
(YES) (N=87)	1.72 ±0.93	29.01±17.73	40.79± 19.71	21.26±12.09	1.36± 0.93	2.13± 1.26		

*The age range of subjects was 20 to 55 years

The p-value of 0.1226 suggests that there is no statistically significant relationship between caffeine intake and semen parameters. This means that the observed differences in semen parameters between the caffeine-consuming group and the non-consuming group could potentially be due to chance alone. Furthermore, the correlation coefficient of 0.9999 indicates a strong positive linear relationship between caffeine intake and semen parameters. This suggests that there was a consistent association between caffeine intake and the observed changes in semen parameters. However, although the correlation coefficient suggests a relationship, the lack of statistical significance as indicates that this relationship may not be meaningful or conclusive. Further research is necessary to determine the true impact of caffeine intake on semen parameters.

5.1.11 Impact of chemicals and Pesticides

Different environmental factors, including different seasons, pesticides, and chemicals, have been examined for their impact on semen quality. The results suggest a relationship between exposure to pesticides and changes in semen parameters. Similarly, the analysis indicates a relation between exposure to chemicals and alterations in semen parameters. These findings highlight the potential influence of environmental factors on male reproductive health and emphasize the need for further studies and awareness regarding the effects of pesticides and chemicals on semen quality.

5.1.11.1 Impact of Pesticides exposer on semen parameters

Groups of participants exposed to pesticides as per table 18, include 121 participants compared with a group of participants without any exposure to pesticides including 884 participants. According to the results, the semen parameters are slightly higher in the group exposed to pesticides had higher values for semen volume (2.30), sperm count (33.58), total motility (45.06), progressive motility (24.40), and morphology (1.68) compared to the non-exposed group. Additionally, the value of the combined defect was lower in the pesticide-exposed group. These results indicate that exposure to pesticides may have a positive impact on semen parameters, indicating the necessity for additional research to comprehensively elucidate the correlation between pesticides and male reproductive health.

Table 18: Impact of Pesticides on semen parameter

Pesticides	**Volume (ml)**	**Sperm count (M/ml)**	**TM (%)**	**PR (%)**	**Morpho (%)**	**Combine defect**	**P-value**	**Correl Coeff.**
(NO) (N=884)	2.07± 1.12	31.18±17.99	42.32± 19.29	22.85± 12.61	1.57± 1.05	2.08± 1.25	0.0387	0.9999
(YES) (N=121)	2.30± 1.38	33.58±17.62	45.06± 19.98	24.40± 13.57	1.68± 1.07	1.90± 1.26		

*The age range of subjects was 20 to 55 years

The p-value of 0.0387 indicates a statistically significant difference in semen parameters between the group exposed to pesticides and the group without exposure. It also suggests that exposure to pesticides has an impact on semen parameters. while the correlation coefficient of 0.9999 indicates a strong positive linear relationship between exposure to pesticides and semen parameters. It shows a consistent association between pesticide exposure and changes in semen parameters. Briefly, the results suggest that exposure to pesticides may have a significant impact on semen parameters. The group exposed to pesticides exhibited slightly higher values for semen parameters compared to the group without exposure. These findings underscore the possible impact of

pesticides on male reproductive health and the necessity for additional research in this domain.

5.1.11.2 Effect of Chemicals exposers on semen parameters

Like pesticides, chemicals are studied for semen analysis, and the results are presented in the table 19 below. According to these results, there is a slight difference in semen parameters between the two groups. The group exposed to chemicals, consisting of 81 participants, is compared to the group without exposure to chemicals, which includes 925 participants. The results show slightly higher values for semen volume, sperm count, total motility, progressive motility, and morphology in the group exposed to chemicals, while PR and volume are not significantly affected compared to the group not exposed to chemicals. However, it is important to note that the observed differences are relatively small. Therefore, these findings suggest a minimal impact of chemicals on semen parameters. While the group exposed to chemicals exhibited slightly higher values for some semen parameters, the differences are not substantial. Further research is necessary to investigate the potential impact of chemicals on semen quality.

Table 19: Impact of Chemicals on semen parameter

Chemicals	Volume (ml)	Sperm count (M/ml)	TM (%)	PR (%)	Morpho (%)	Combine defect	P-value	Correl Coeff.
(NO) (N=925	2.10 ±1.15	31.38±17.85	42.51± 19.17	23.02±12.60	1.57± 1.03	2.05± 1.24	0.0658	0.9999
(YES) (N=81)	2.12 ±1.26	32.46±19.11	44.20± 21.77	23.14±14.20	1.75± 1.20	2.06± 1.46		

*The age range of subjects was 20 to 55 years

The p-value of 0.0658 suggests that there is no statistically significant difference in semen parameters between the group exposed to chemicals and the group without exposure to chemicals. In comparison to a significance as level of 0.05, a p-value of 0.0658 is slightly higher. It suggests that there is a relatively small probability (6.58%) that the observed differences in semen parameters between the two groups occurred by

chance. However, the p-value still indicates a trend or tendency towards a difference. The correlation coefficient of 0.9999 indicates a strong positive linear relationship between exposure to chemicals and semen parameters, suggesting that there is a consistent association between the two. In conclusion, the results do not provide strong evidence for the significant impact of exposure to chemicals on semen parameters. Further research is needed to investigate this relationship more comprehensively.

5.1.12 Heatmap of multivariate coefficient

This represents (see figure 35) the a heatmap illustrating multivariate correlation coefficient between semen parameters and various lifestyle or physiological factors.

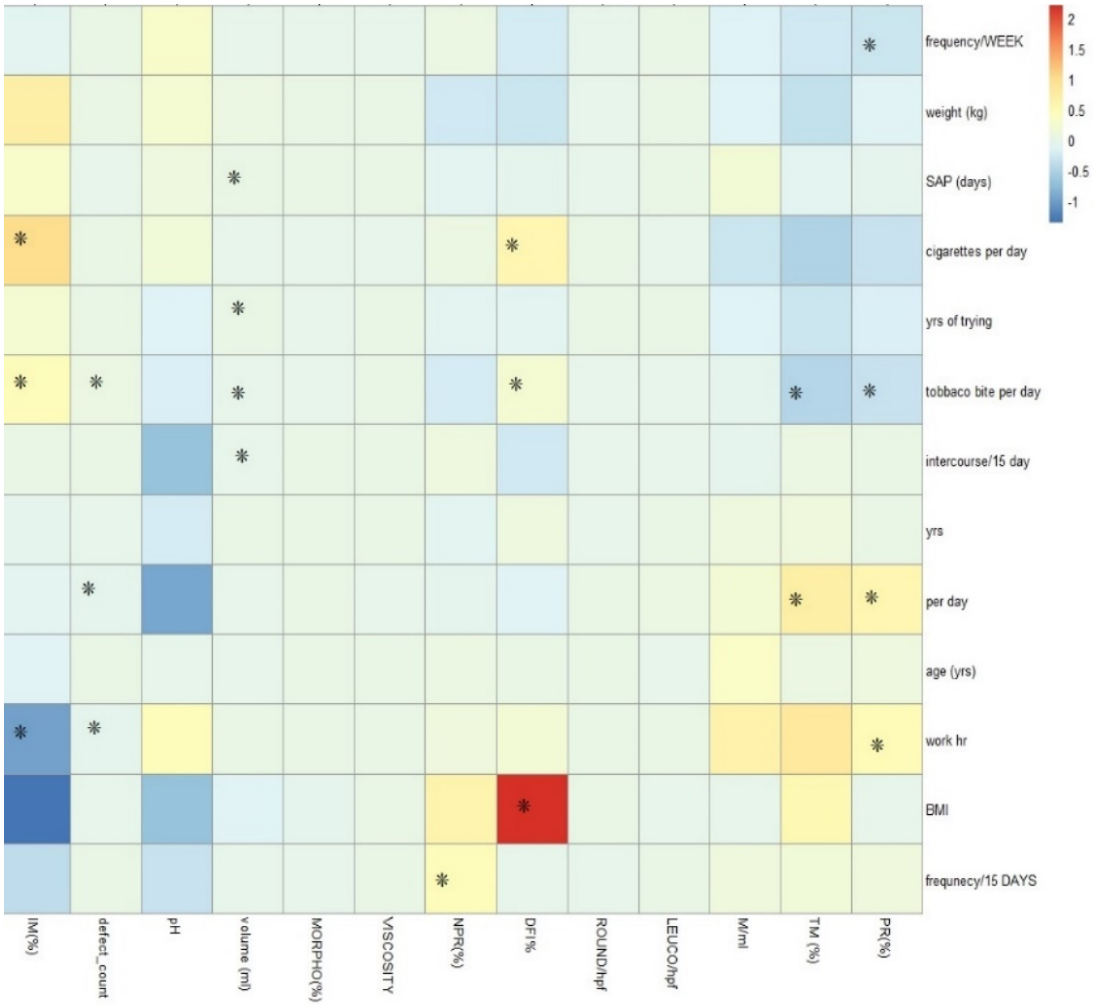


Figure 35: Heatmap for multivariate coefficients showing significant relationships among the variables

The colour scale indicates the strength and direction of correlation, with red showing positive and blue showing negative association (The strength of the predictors positive and negative influence on the outcome is represented by the colour scale at the top right corner of the figure). Asterisk (*) denote statically significant correlations. Notably, DFI shows strong positive correlation with age, BMI and tobacco use, and negative correlation with key semen parameter like motility and morphology. Other factors such as SAP also display significant associations with semen quality. This heatmap highlights the complex, multifactorial influences on male fertility.

5.2 Molecular Aspects of semen quality in terms of DFI

We conducted a comprehensive analysis of all factors to examine their relationship with DFI. This evaluation aimed to establish correlations between these risk factors and DFI, providing insights into their impact on semen quality. The data derived from these analysed samples was presented in each following tables and figures with the corresponding number of samples of each group.

These all tables include numerical values preceding the "±" sign, which represents the mean average, while the digits following the sign represent the standard deviation. DFI values are expressed as percentages. The DFI results were obtained and analysed in relation to various risk factors, including health, lifestyle, diet, and environmental factors. The data revealed significant correlations between DFI and these factors, specifically in individuals with a history of smoking, advanced age, exposure to environmental contaminants, and certain medical conditions. These findings highlight the potential impact of these risk factors on sperm DNA integrity and suggest their association with increased DNA fragmentation. They provide valuable insights into the underlying mechanisms and implications of these associations within the context of male fertility and reproductive health.

It is important to note that DFI values below 30% are considered indicative of good semen quality, while DFI values above this threshold are associated with abnormal sperm (see table 20). These results serve as a valuable reference for evaluating sperm

quality and assessing the potential effects of risk factors on DNA fragmentation, ultimately aiding in the comprehension and administration of infertility

Table 20: DFI range indicating sperm quality

DFI Value	**Sperm quality**
DFI<15%	Very Good Sperm
15% < DFI <30%	Good Sperm
DFI > 30%	Abnormal Sperm

Various risk factors have been evaluated to investigate their impact on semen quality, particularly in terms of the DFI. Samples for each risk factor are primarily categorized into two groups: one where the risk factor is present (YES) and another where the risk factor is absent (NO), with n samples among a total sample. Furthermore, the samples are further categorized into different levels based on factors such as age, BMI, consumption of different diets (e.g., caffeine), and addictions. By comparing these results, we appear to establish the relationship between different risk factors and their impact on semen quality.

5.2.1 Impact of age on DNA fragmentation

First, we categorized the semen samples into six age groups to analyse the impact of age on the DFI. The groups were defined as age below 25 years, between 26-30 years, 31-35 years, 36-40 years, 41-45 years, and above 45 years.

As per the values mentioned in the figure 36, it appear to conclude that the DFI value was directly proportional to the age factor. The age group of >30 years was identified as a causative factor for higher DFI values. Individuals in this age group exhibit DFI values exceeding 30%, indicating the presence of abnormal sperm. This emphasizes the impact of age on DFI and suggests that advanced age may adversely affect semen quality, specifically in terms of DNA integrity. The correlation coefficient of 0.07 indicates a positive linear relationship between age and DFI, suggesting that there is a consistent association between these two. In conclusion, the results do provide strong

evidence for the significant impact of age on semen parameters. Further research is needed to investigate this relationship more comprehensively.

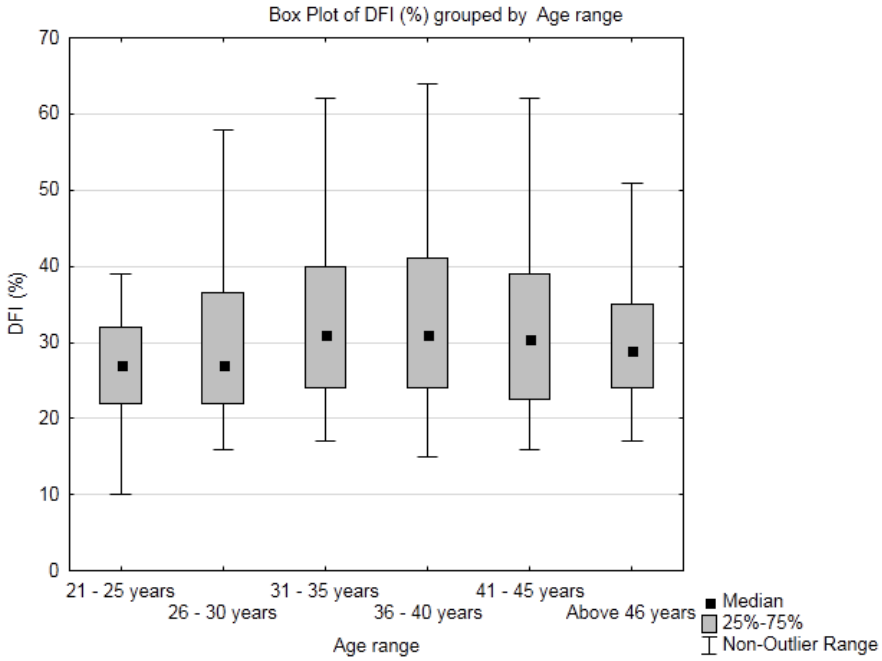


Figure 36: DFI percentage in different age groups

5.2.2 BMI and DNA quality of sperm

The BMI was categorized into underweight, normal, overweight, and obese to observe the impact of obesity on semen quality. A BMI value between 18.5 and 24.9 kg/m^2 is considered within the normal weight range, while values above 25 and below 30 are classified as overweight.

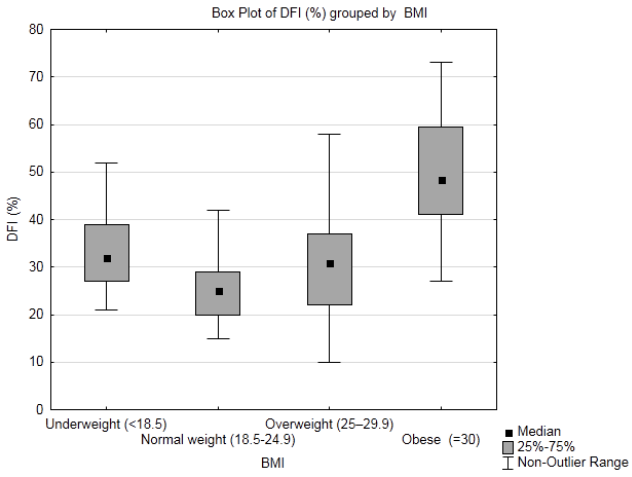


Figure 37: DFI percentage in different BMI groups

BMI values below the normal weight range indicate underweight, while values above 30 indicate obesity. Based on the values presented in the figure 37, it appears to be concluded that the highest DFI value (50.06) is obtained in patients with an obese BMI, indicating an increased risk. The DFI value was found to be lower (25.99) in the normal weight BMI group, while it was higher in the underweight (33.56) and overweight (30.88) BMI groups. These findings suggest a potential association between obesity and increased DNA fragmentation, highlighting the importance of BMI as a factor influencing semen quality. The correlation coefficient of 0.56 represent the strong positive linear relationship and concluded that there is a consistent association between BMI and DFI.

5.2.3 Impact of Lifestyle Factors on semen parameters

5.2.3.1 Cigarette smoking

The groups of smoking and non-smoking participants were compared to assess their performance in terms of the DFI. The results indicated higher DFI values in the smoking group compared to the non-smoking group, highlighting the negative impact of cigarette smoking on semen quality. However, it was important to note that both groups exhibited abnormal sperm as indicated by DFI values exceeding the significant level of 30%.

Table 21: Impact of cigarette smoking on DFI

	Smoking habit Vs DFI (%)			
	No. of participants	Average	Std. Deviation (±)	p value
Smoker	105	36.29	15.48	0.00
Non-smoker	425	31.81	11.98	

*The age range of subjects was 20 to 55 years

These data in the table 21, indicate that cigarette smoking adversely affects sperm quality, as demonstrated by elevated DFI values. The existence of defective sperm in both cohorts underscores the necessity to examine the influence of smoking on semen quality. Furthermore, as per figure 38, smoking consumption was evaluated for 0, 1-5, 6-10 and more than 10 cigarettes per day. The results, shown lowest DFI values in

nonsmoker participants. However, it was increasing as frequency of smoking in increased.

In contrast, the other groups exhibit higher DFI values, with the group consuming more than 1-10 cigarettes per day showing a significant increase in DFI values. Considering these data, one appears to infer that higher levels of cigarette smoking have a greater impact on DFI and, consequently, on semen quality.

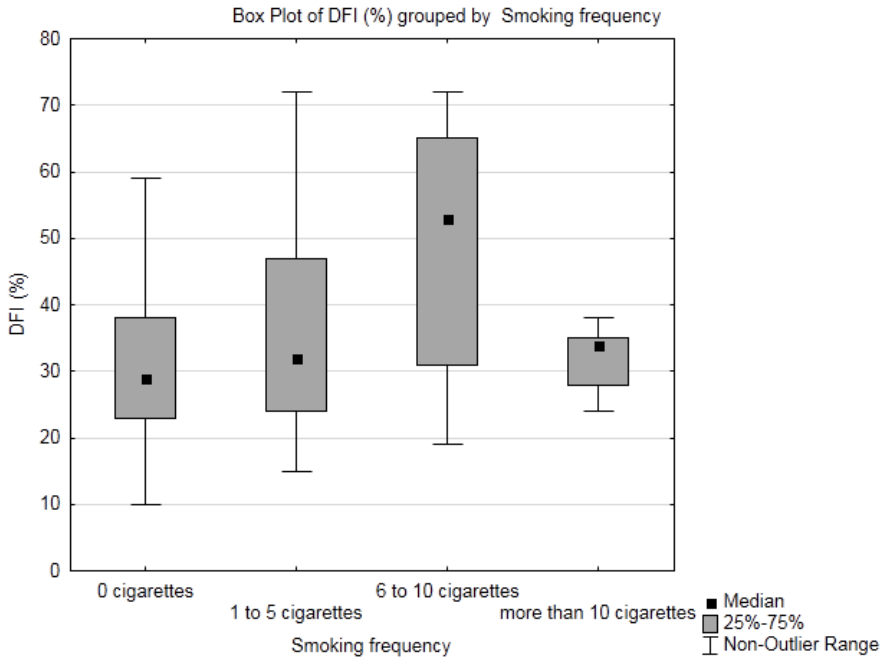


Figure 38: DFI percentage against smoking groups

The significant increase in DFI values in the group consuming more than 5 cigarettes per day indicates a negative effect on sperm health, shown in figure 38. Therefore, reducing, or quitting cigarette smoking is crucial for improving semen quality and overall fertility outcomes.

5.2.3.2 Impact of Tobacco consumption DNA injury

According to the below table 22 and figure 39, there was a slight difference in DFI values between the groups that consume tobacco and those that do not consume tobacco. The slightly higher DFI in the tobacco-consuming group indicates a negative

impact of tobacco consumption on semen quality. Both groups exhibit DFI values higher than the significant level, indicating the presence of abnormal sperm.

Table 22: Impact of tobacco consumption on DFI

Tobacco use Vs DFI (%)				
	No. of participants	Average	Std. Deviation (±)	p value
Yes	131	33.06	12.38	0.71
No	399	32.58	13.03	

*The age range of subjects was 20 to 55 years

The table 22, revealed that the level of tobacco consumption has a variable impact on DFI. The group consuming tobacco >5-10 times per day shows higher DFI values, while an increase in consumption beyond 1 time per day results in a decline in DFI percentage. However, as per results it was essential to acknowledge that all the groups, ranging from no tobacco consumption to more than 10 time per day, demonstrate the presence of abnormal sperm due to average DFI values exceeding the significant level. DFI value is extremely higher in the group consuming tobacco more than 10 times per day and it shows significant impact of tobacco consumption on DFI appears to be observed based on these findings.

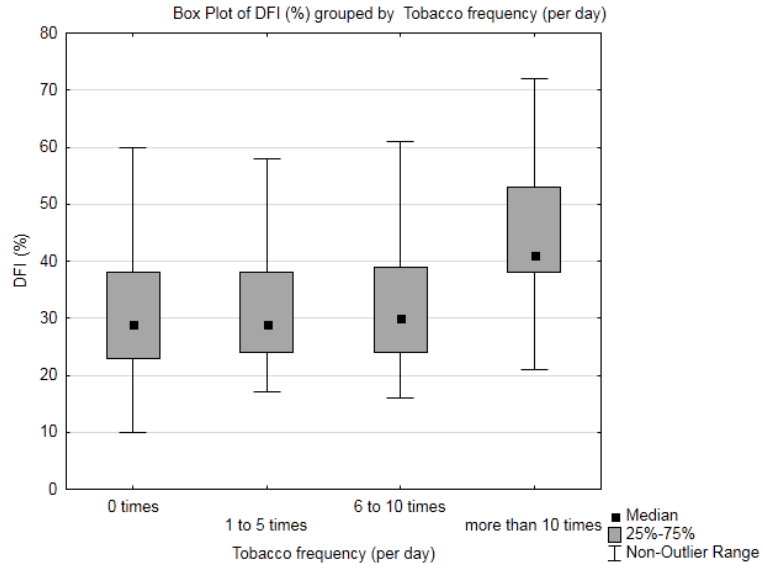


Figure 39: DFI percentage against tobacco consumption in groups

5.2.3.3 Combine impact of Tobacco consumption and Cigarette smoking on DFI

The combined effect of tobacco consumption and cigarette smoking has a significantly impacted on DFI values (see table 23). The group of participants who both consume tobacco and smoke cigarettes (20 participants) exhibits considerably higher DFI values compared to the group of non-consumers (170 participants). The presence of higher DFI values in both groups indicates the presence of abnormal sperm. These findings suggest that the combined effect of tobacco consumption and cigarette smoking negatively affects semen quality, as indicated by the increased DFI values.

Table 23: Combine impact of tobacco consumption, and cigarette smoking on DFI

Tobacco, smoking habit Vs DFI (%)				
	No. of participants	Average	Std. Deviation (±)	p value
Yes	20	41.83	15.75	0.06
No	170	34.97	12.56	

*The age range of subjects was 20 to 55 years

5.2.3.4 Alcohol consumption

The impact of alcohol consumption on DFI values was assessed, comparing a group of 203 participants who consume alcohol to a group of 327 participants who do not consume alcohol. Therefore, it appears to be concluded that the impact of alcohol consumption on semen quality was significant, with higher DFI values observed in the alcohol-consuming group.

From the results displayed in above figure 40, it appears to be observed that there was an increase in DFI values with higher levels of alcohol consumption. The results shown in figure 40, a slight increase in DFI values in the alcohol-consuming group, suggesting an adverse effect of alcohol on semen quality. However, it was important to note that both groups exhibited DFI values higher than the significant threshold, indicating the presence of abnormal sperm.

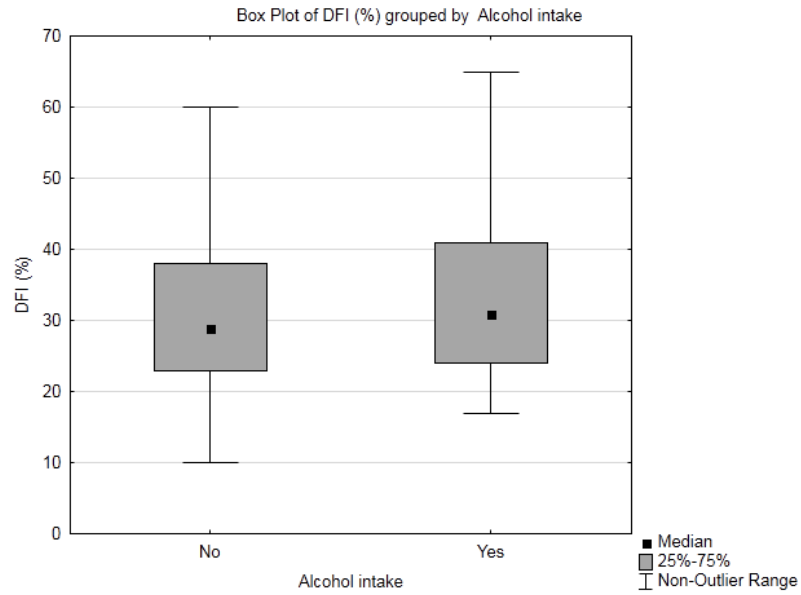


Figure 40: DFI percentage against alcohol consumption

5.2.4 Effect of Medical History on semen parameters

The figure 41, presented the impact of medical history on DFI values. The group with no medical history, including 400 samples, shown an average DFI of 33.05 % with a standard deviation of 12.48, On the other hand, the group with a medical history, consisting of 130 samples, exhibits a slightly lower average DFI of 32.58% with a standard deviation of 12.99

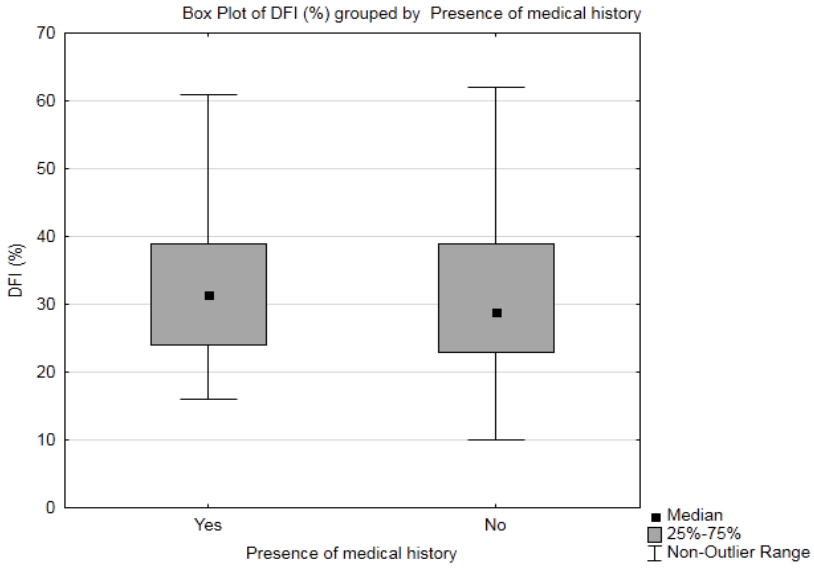


Figure 41: DFI percentage against medical history

These findings suggest that individuals with no medical history have slightly lower DFI values, indicating potentially better semen quality compared to those with a medical history. However, DFI values in both groups exceed 30%, indicating the presence of abnormal sperm. Therefore, while there may be a slight difference, there is no significant (p-value =0.72) impact of medical history on semen quality based on the observed results.

5.2.5 Impact of Veg and Non-veg Diet on DFI

The figure 42 presented the impact of diet on DFI values. The group following a vegetarian diet (n=39) has an average DFI of 32.92% while the group following a non-vegetarian diet (n=139) exhibits a slightly higher average DFI of 35.54%. These results suggested that individuals following a non-vegetarian diet may have slightly higher DFI values, indicating potentially poorer semen quality compared to those following a vegetarian diet.

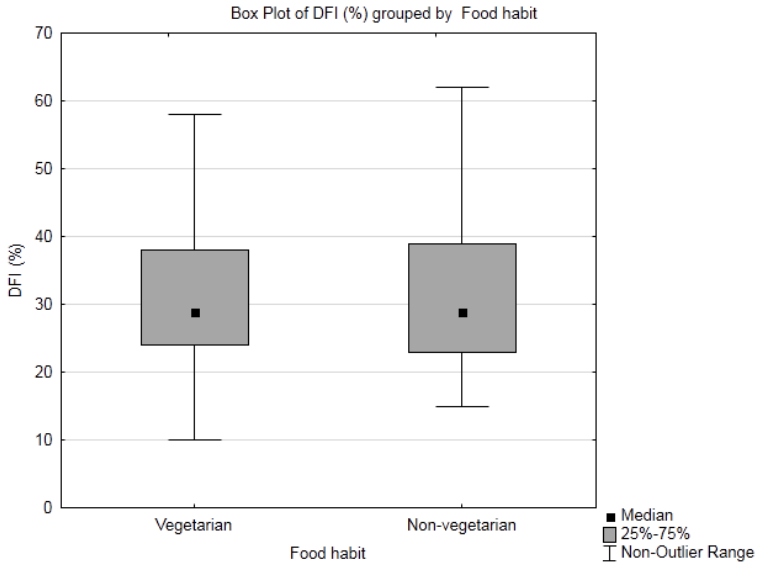


Figure 42: DFI percentage against Diet type

However, it is important to note that the difference in DFI values between the two groups is relatively small, and both groups show DFI values above the normal range. Therefore, diet may not have some influence on DFI with other factors contributing to overall semen quality.

5.2.6 Use of filtered water and non-filtered water vs DFI

The impact of using filtered water on DFI values against non-filtered water displayed in the figure 43. The group that does not use water filters consists of 199 participants and shows a DFI of 32.95% while the group that uses water filters consists of 331 participants, and shows a DFI of 35.54%. From these results, it appears to be observed that there is a slight difference in DFI values between the two groups. The group using water filters shows a slightly higher average DFI compared to the group that does not use water filters. However, both groups have DFI values exceeding 30%, indicating the presence of abnormal sperm. Consequently, these data indicate that the utilization of water filters does not significantly affect DFI values or semen quality. The observed difference between the two groups is relatively small and may not be clinically significant

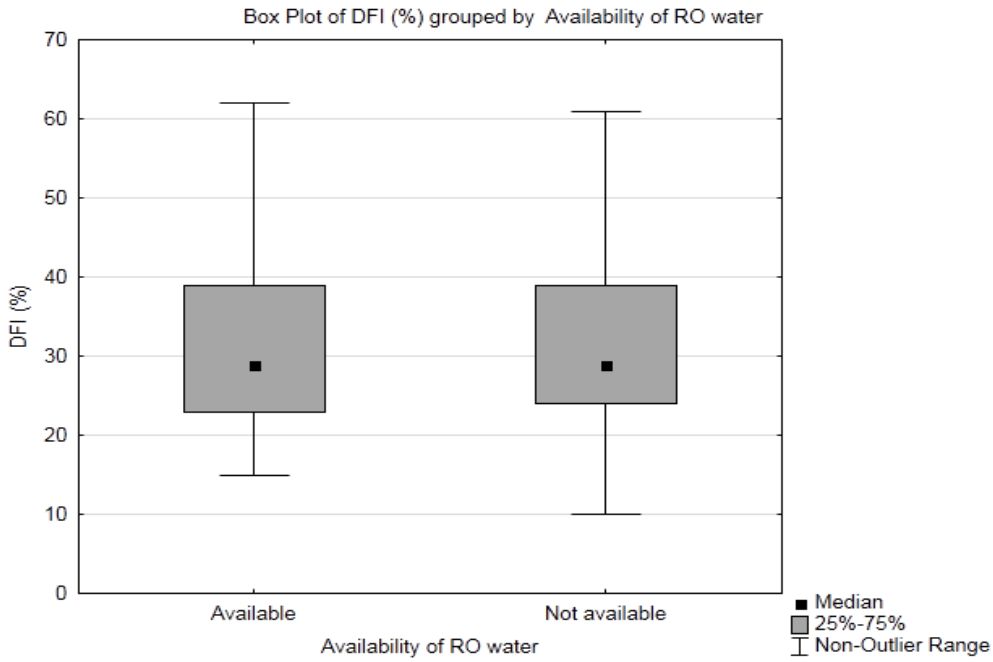


Figure 43: DFI percentage against filter water consumption

5.2.7 Effect of Fast Food on DNA fragmentation

The table 24, presented the impact of fast-food consumption on the DFI. The results include groups divided based on individuals consuming or not consuming fast food. For the non-consumer group, the average DFI was shown as 31.26% with a standard

deviation of 11.95. Instead, the fast-food consumer group exhibits a slightly higher DFI at 37.89% with a standard deviation of 14.62, however it was clinically non-significant.

Table 24: Impact of fast food on DFI

	Fast Food habit Vs DFI (%)			
	No. of participants	Average	Std. Deviation (±)	p value
NO	415	31.26	11.95	0.10
YES	115	37.89	14.62	

*The age range of subjects was 20 to 55 years

According to the results, individuals consuming fast food had a slightly higher DFI compared to those who do not consume fast food. The difference in DFI values between the two groups indicates a potential association between fast food consumption as well as augmented DNA fragmentation in sperm cells. The results raise awareness about the potential impact of dietary habits, such as fast-food consumption, on male fertility and reproductive health. However, an underlying relationship amongst fast food consumption and SDF needs comprehensive study.

5.2.8 Food Supplement and DNA fragmentation

The table 25, shown the impact of food supplements on DFI values. The group that does not consume food supplements consists of 488 participants, with an average DFI of 32.32%. While the group that consumes food supplements consists of 42 participants, with an average DFI of 37.10%.

Table 25: Impact of food supplement on DFI

	Food supplement Vs DFI (%)			
	No. of participants	Average	Std. Deviation (±)	p value
NO	488	32.32	12.69	0.04
YES	42	37.10	14.12	

*The age range of subjects was 20 to 55 years

The results shown a notable difference in DFI values between the two groups with higher DFI in the group consuming food supplements. Additionally, the standard deviation in the food supplement group was also higher, indicating a greater variation in DFI values within that group. These findings lead to the conclusion that consumption

of food supplements may have a negative impact on DFI values and potentially on semen quality.

The group consuming food supplements exhibits higher DFI values, which suggests a higher presence of abnormal sperm. Overall, these results indicate a potential association between the use of food supplements and increased DFI values.

5.2.9 Effect of caffeine via Tea/coffee on DFI

Consumption of Tea/coffee was compared for impact on DFI against the non-consuming group. The table 26 shows no significant difference in DFI for the group consuming Tea/coffee (460 participants) and the group not consuming (70 participants). However, both group shows a DFI percentage higher than the significant value indicating the presence of abnormal sperm. Thus, it shows no significant impact on semen quality.

Table 26: Impact of tea/coffee consumption on DFI

	Caffeine intake Vs DFI			
	No. of participants	Average	Std. Deviation (±)	p value
Yes	460	32.80	13.03	0.63
No	70	32.01	11.73	

*The age range of subjects was 20 to 55 years

The comparison of different groups consuming an increasing level of tea/coffee per day shows the highest DFI value (34.56) in the group consuming tea/coffee more than 6 times per day. On the other hand, figure 44, shown the group consuming tea/coffee 1-5 times per day (32.67) or not consuming tea/coffee (32.01) shows slight differences in DFI values. The group consuming tea/coffee more than 10 times per day exhibits a declined value of DFI (27.00), indicating variability in performance as it included only 4 subjects.

These findings lead to the conclusion that an increasing level of tea/coffee consumption may have an impact on DFI values and potentially on semen quality. The group with higher tea/coffee consumption shows higher DFI values, suggesting a higher presence of abnormal sperm. Nonetheless, it is crucial to acknowledge that the disparities in DFI

values between the groups are relatively small. Therefore, it needs further research with larger sample sizes for a more definitive conclusion concerning the influence of tea and coffee consumption on DFI and semen quality.

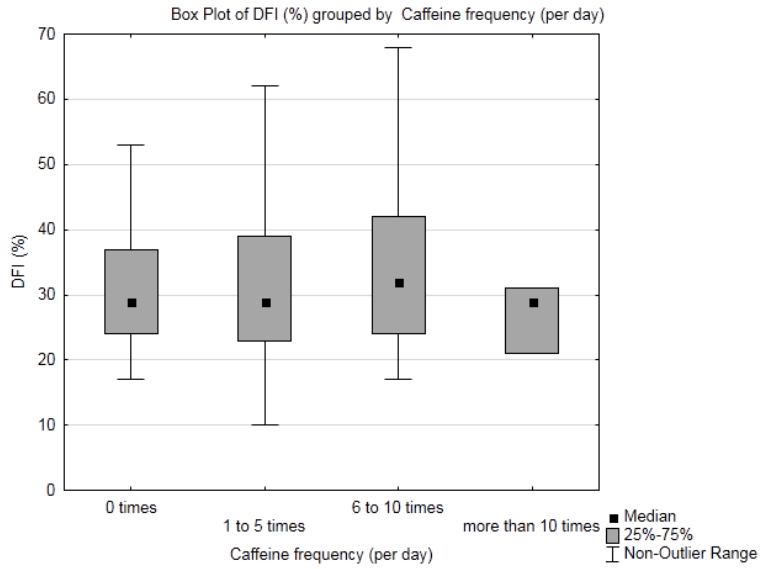


Figure 44: DFI percentage against tea/coffee consumption in groups

5.2.10 Effect of carbonated drink consumption on DNA damage

The table 27, presented the impact of cold drink consumption on DFI values. The group that does not consume fizzy drinks (480 participants), shows a DFI of 32.22% and a standard deviation of 12.27. On the other hand, the group that consumes cold drinks (50 participants), shows a higher DFI of 37.26% and a higher standard deviation of 17.04

Table 27: Impact of cold drink consumption on DFI

	fizzy Vs DFI			
	No. of participants	Average (%)	Std. Deviation (±)	p value
Yes	50	37.26	17.04	0.07
No	480	32.22	12.27	

*The age range of subjects was 20 to 55 years

As per the results show a significant difference in DFI values between the two groups, with the group consuming cold drinks exhibiting higher DFI values. This suggests a potential negative impact of cold drink consumption on semen quality, as higher DFI values are associated with a higher presence of abnormal sperm. However, the difference in DFI values and standard deviation between the two groups is relatively large, indicating a greater variation in DFI values within the group. These findings lead to the conclusion that the consumption of cold drinks may have a negative impact on DFI values and potentially on semen quality. The group consuming cold drinks shows higher DFI values, indicating a potential association between cold drink consumption and increased DNA fragmentation in sperm.

5.2.11 Impact of Caffeine intake (tea/coffee and Cold Drink) on DFI

The combined effect of caffeine intake from tea/coffee and cold drink consumption on DFI values has shown in table 28. The group that does not consume caffeine from tea/coffee and cold drinks (67 participants), shows an average DFI of 31.64% and a standard deviation of 11.64. On the other hand, the group that consumes caffeine from tea/coffee and cold drinks (47 participants) shows an average DFI of 37.06% and a higher standard deviation of 17.36.

Table 28: Impact of caffeine intake on DFI

Cold drink Vs DFI				
	No. of participants	Average (%)	Std. Deviation (±)	p value
Yes	47	31.64	11.64	0.04
No	67	37.06	17.36	

*The age range of subjects was 20 to 55 years

The results shown a significant difference in DFI values between the two groups, with the caffeine-consuming group exhibiting a substantially higher DFI value compared to the non-consuming group. This indicates that caffeine intake has a negative effect on semen quality, showing a higher presence of abnormal sperm.

The difference in mean DFI value and the higher standard deviation in the caffeine-consuming group further emphasize the impact of caffeine intake on DFI. These

findings suggest that caffeine consumption contribute to increased DNA fragmentation in sperm, indicating significant negative effects on semen quality.

5.2.12 Impact of Pesticides exposer

The results table 29 shows the impact of pesticides on DFI values. The group with no pesticide exposure consists of 473 participants, with an average DFI of 32.83% while the group with pesticide exposure, consisting of 57 participants, has an average DFI of 31.60%. These findings lead to the conclusion that there is a slight difference in DFI values between the group with no pesticide exposure and the group with pesticide exposure.

Table 29: Impact of pesticides on DFI

	Pesticides Vs DFI			
	No. of participants	Average (%)	Std. Deviation (±)	p value
Yes	57	31.60	11.83	0.70
No	473	32.83	12.98	

*The age range of subjects was 20 to 55 years

The group without pesticide exposure shows a slightly higher average DFI compared to the group with pesticide exposure. However, both groups exhibit DFI values above the significant threshold (30%), indicating the presence of abnormal sperm in both cases. Therefore, while there was a small difference in DFI values between the two groups, it is not be statistically significant. Both groups demonstrate abnormal sperm presence based on DFI values.

5.2.13 Impact of Chemicals exposers on DFI

The impact of chemicals on DFI appears to be compared within groups with and without chemical exposure. A group exposed to chemicals (34 participants) shows less DFI of 28.56% as compared to the group not exposed to chemicals (496 participants), with an average DFI of 32.98% has shown table 30. These findings lead to the conclusion that there was a notable difference in DFI values between the group with

and without chemical exposure. The group without chemical exposure exhibits a higher average DFI compared to the group with chemical exposure.

Table 30: Impact of chemicals on DFI

	Pesticides Vs DFI			
	No. of participants	Average (%)	Std. Deviation (±)	p value
Yes	34	28.56	10.86	2.1
No	496	32.98	12.95	

*The age range of subjects was 20 to 55 years

Additionally, the standard deviation in the chemical exposure group is lower, indicating less variation in DFI values within that group. The lower DFI values in the group with chemical exposure suggest a potential positive impact of chemicals on semen quality. However, both groups show DFI values above the significant threshold (30%), indicating the presence of abnormal sperm in both cases. Therefore, while there was a significant difference in DFI values between the two groups, it needs to interpret these findings with further research and analysis to understand the specific chemicals involved, their concentrations, and their potential effects on DFI and overall semen quality.

5.2.14 Microphotograph of Results of SCD Glass slide

All microphotographs in figure 45 shows outcome of SCD test used to asses DFI by visualizing halo pattern. Sperm with intact DNA exhibit large or medium halos, while those with fragmented DNA display small or no halos. Each sample (a to k) is labelled with its corresponding DFI percentage, indicating the proportion of sperm with fragmented DNA. For example, sample j (DFI 59%) shows more sperm with small/no hallos, reflecting higher DNA damage, whereas sample i (DFI 24%) shows a greater number of large halos, indicating better DNA integrity. This visual assessment helps quantify DNA fragmentation and its variation across different individuals. All micrograph were clicked using an inverted microscope (Axio Vert.A1, Carls Zeiss Co. Ltd.) with 40x objective.

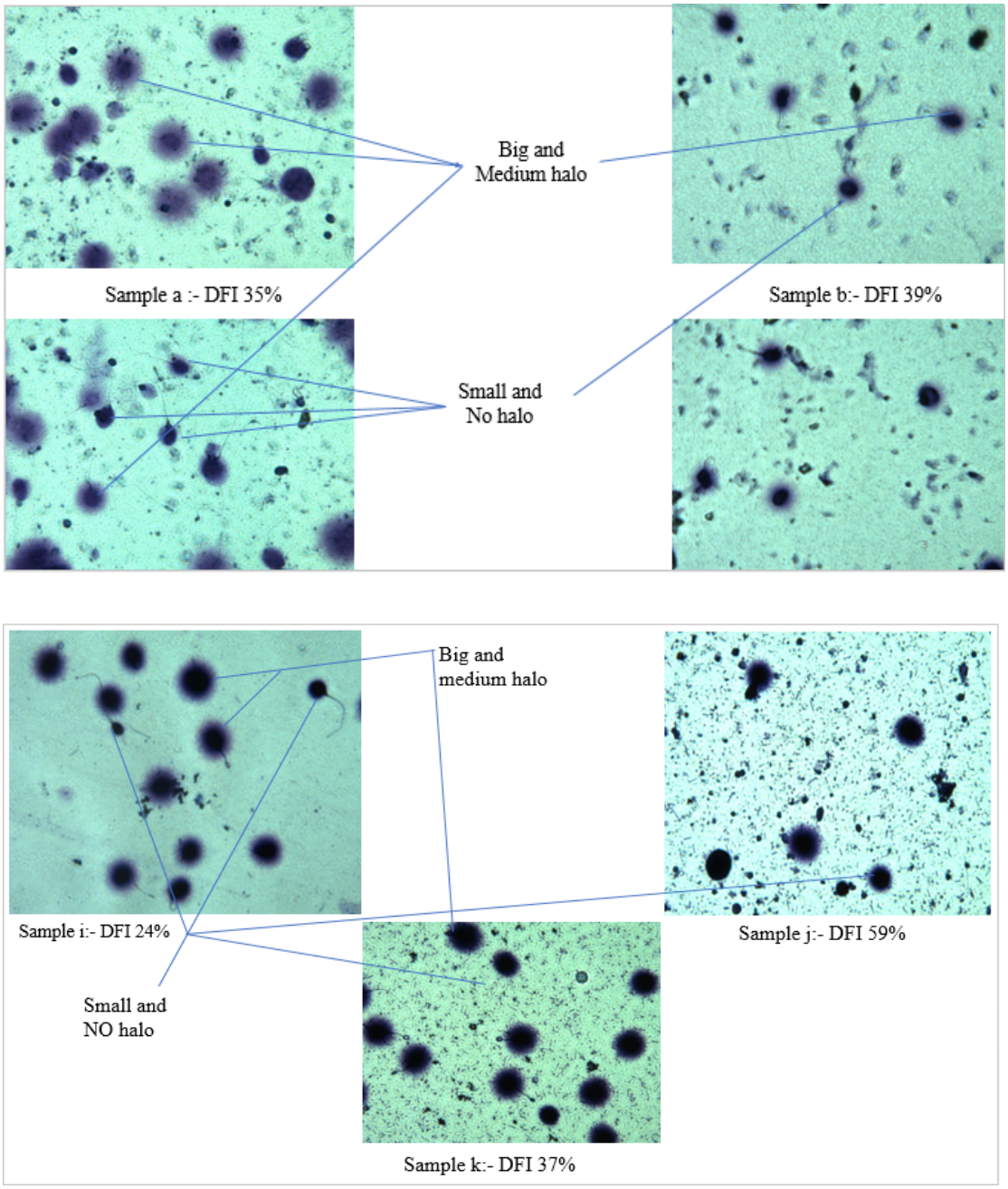


Figure 45: SCD test outcomes indicating variety of DNA fragmentation including halo

5.3 Impact of Sexual abstinence period on semen quality

The effect of SAP on semen parameters appears to vary depending on the specific context and underlying factors was shown in figure 46 and 47. SAP refers to the

clumping or sticking together of sperm cells, which appears to affect their motility and overall function. In general, a higher percentage of sperm agglutination appears to be associated with reduced sperm motility, which may negatively impact semen parameters such as total motility (TM) and progressive motility (PR).

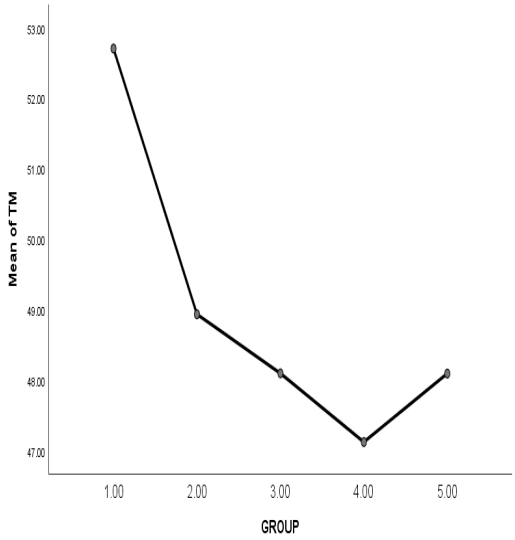


Fig 46 a : Mean plot of Count vs SAP

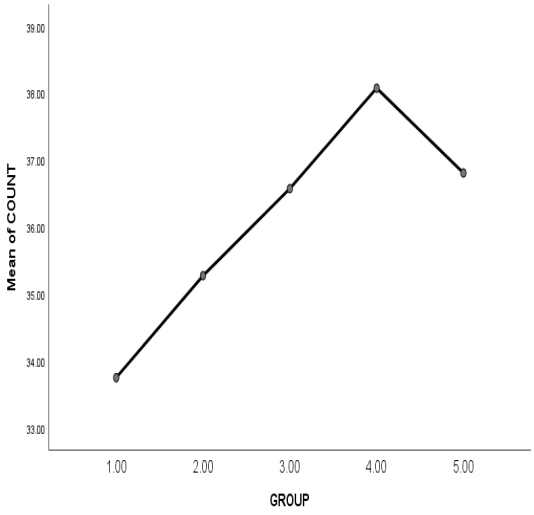


Fig 46 b: Mean plot of Total Motility vs SAP

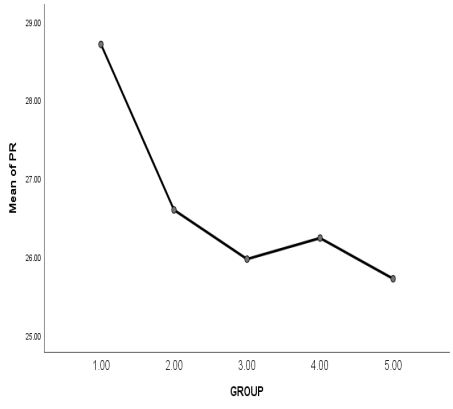


Fig 46 c: Mean plot of Progressive Motility vs SAP

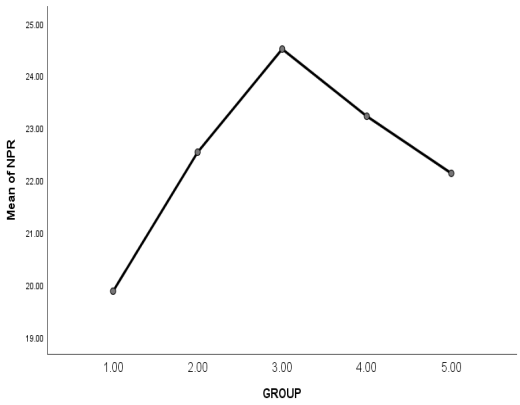


Fig 46 d: Mean plot of non-Progressive Motility vs SAP

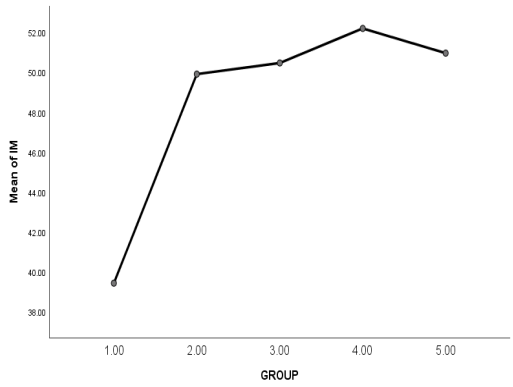


Fig 46 e: Mean plot of Immotile sperms vs SAP

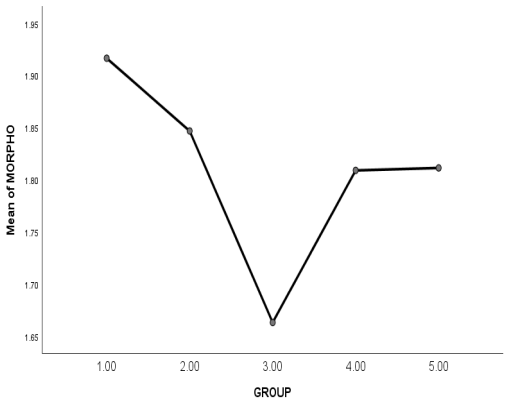


Fig 46 f: Mean plot Morphology vs SAP

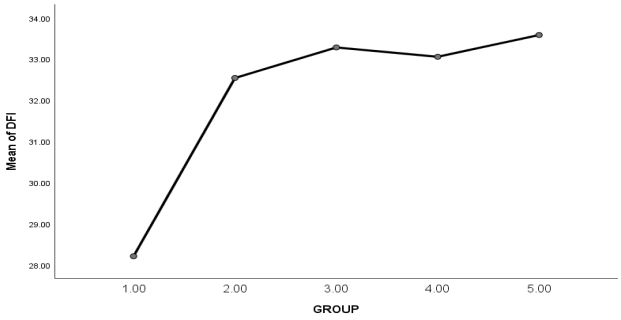


Fig 46g: Mean plot of DFI vs SAP

Figure 46: SAP vs semen parameters, age range 20 to 55 years (a) represents the impact of SAP on sperm count, (b) represents the impact of SAP on total motility, (c) represents the impact of SAP on progressive motility, (d) represents the impact of SAP on immobility, (e) represents the impact of SAP on morphology, (f) represents the impact of SAP on DFI

It appears to also affect sperm count and morphology to some extent. The one-way ANOVA was performed for Count, Total Motility, Progressive Motility, Non-Progressive Motility, Immotile sperms, Morphology and DFI with patients with different Sexual Abstinence Period (SAP) and no significant differences were observed except in immotile group.

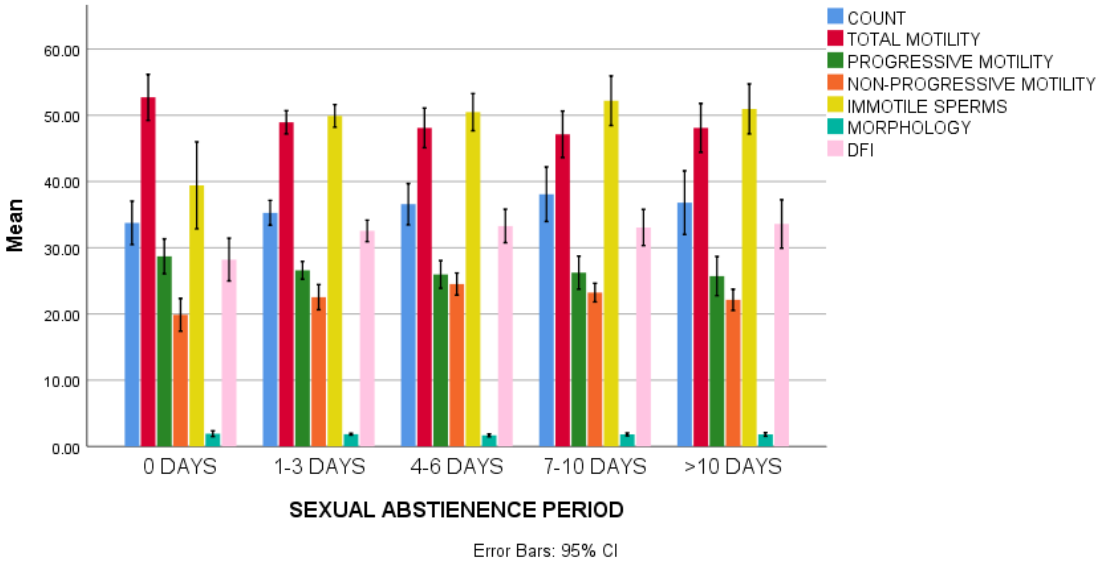


Figure 47: Simple bar graph of Mean of Count, Total Motility, Progressive Motility, Non-Progressive Motility, Immotile sperms, Morphology, DFI by group by index

The maximum sperm count was observed in SAP of 7-10 days category among all the groups whereas the least sperm count was observed in SAP of 0 days but had no statistically significant Figure 47 present a bar graph showing the mean values of various semen parameters sperm count, total motility, PR motility, non-PR motility, morphology and DFI.

Across different SAP show that motility (TM and PR) tends to decline with increasing days of abstinence. While immotile sperm and DFI gradually increases, especially beyond 7 days. Sperm morphology remains relatively stable, with a slight drop at longer abstinence period. The optimal semen quality appears between 1-3 days of abstinence. Table no 31 explained the all-statistical data of impact of SAP on semen parameters.

Table 31: SAP vs semen parameters

		No	**Mean**	**Std. Devi.**	**Std. Error**	**95% Confidence Interval for Mean**	
						Lower Bound	Upper Bound
COUNT M/ml	SAP 0 days	24	33.75	7.79	1.59	30.45	37.04
	SAP 1-3 days	222	35.27	14.21	.95	33.39	37.15

	SAP 4-6 days	92	36.57	15.09	1.57	33.45	39.70
	SAP 7-10 days	89	38.07	19.58	2.07	33.95	42.20
	SAP >10 days	53	36.81	17.44	2.39	32.00	41.61
	Total	480	36.13	15.63	.713	34.73	37.53
TM (%)	SAP 0 days	24	52.70	8.25	1.68	49.22	56.19
	SAP 1-3 days	222	48.93	13.27	.89	47.18	50.69
	SAP 4-6 days	92	48.09	14.47	1.50	45.10	51.09
	SAP 7-10 days	89	47.12	16.73	1.77	43.59	50.64
	SAP >10 days	53	48.03	13.41	1.84	44.39	51.79
	Total	480	48.53	14.02	.64	47.27	49.79
PR (%)	SAP 0 days	24	28.70	6.25	1.27	26.06	31.35
	SAP 1-3 days	222	26.59	10.07	.67	25.26	27.92
	SAP 4-6 days	92	25.96	10.12	1.05	23.87	28.06
	SAP 7-10 days	89	26.23	11.82	1.25	23.74	28.72
	SAP >10 days	53	25.71	10.74	1.47	22.75	28.67
	Total	480	26.41	10.33	.47	25.48	27.34
NPR (%)	SAP 0 days	24	19.87	5.85	1.19	17.40	22.34
	SAP 1-3 days	222	22.53	14.37	.96	20.63	24.43
	SAP 4-6 days	92	24.51	8.08	.84	22.83	26.18
	SAP 7-10 days	89	23.22	6.70	.71	21.81	24.63
	SAP >10 days	53	22.13	5.80	.79	20.53	23.73
	Total	480	22.86	11.06	.50	21.87	23.85
IM (%)	SAP 0 days	24	39.41	15.55	3.17	32.84	45.98
	SAP 1-3 days	222	49.90	12.98	.87	48.19	51.62
	SAP 4-6 days	92	50.46	13.59	1.41	47.65	53.28
	SAP 7-10 days	89	52.20	17.79	1.88	48.45	55.95
	SAP >10 days	53	50.96	13.71	1.88	47.18	54.74

	Total	480	50.03	14.48	.66	48.73	51.33
MORPHO (%)	SAP 0 days	24	1.91	1.017	.20	1.48	2.34
	SAP 1-3 days	222	1.84	1.094	.073	1.70	1.99
	SAP 4-6 days	92	1.66	1.051	.10	1.44	1.88
	SAP 7-10 days	89	1.80	1.021	.10	1.59	2.02
	SAP >10 days	53	1.81	0.98	.13	1.54	2.08
	Total	480	1.80	1.05	.04	1.70	1.89
DFI (%)	SAP 0 days	24	28.20	7.65	1.56	24.97	31.44
	SAP 1-3 days	222	32.54	12.31	.82	30.91	34.16
	SAP 4-6 days	92	33.28	12.22	1.27	30.74	35.81
	SAP 7-10 days	89	33.05	12.98	1.37	30.32	35.79
	SAP >10 days	53	33.58	13.31	1.82	29.91	37.25
	Total	480	32.67	12.35	.56	31.56	33.78

*The age range of subjects was 20 to 55 years

5.4 Impact of Seasons on semen parameters

As there is variation in temperature during different seasons which may impact on semen parameters. For comparing the average values of the semen parameters according to the four seasons, we performed an ANOVA test. No statistically significant variation was observed in the mean volume between seasons ($p > 0.05$), whereas count, total motility, progressive motility, and morphology showed a significant variation during different seasons ($p < 0.01$). A downtrend in all three semen parameters from summer to monsoon appears to be seen, whereas there was a clear uptrend. The overall average quality of semen, according to the data considering all these parameters, appears to be higher and improved in the winter season compared to the monsoon season (Table 32).

Table 32: Descriptive of seasonal variations in semen parameters

Sperm parameters	Summer (N = 269)	Monsoon (N = 406)	Autumn (N = 357)	Winter (N = 313)	F	p-value
	Mean ± SD	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**		
Volume (ml)	1.97 ±0.91	2.05 ± 0.92	2.14 ± 0.91	2.11 ± 0.83	2.077	0.101
Count (M/ml)	48.02 ±20.98	46.87 ± 20.44	48.11 ± 22.29	52.35 ± 21	4.302	0.005
Total motility (%)	43.82±13.62	41.31 ± 17	44.97 ± 16.27	47.32 ± 14.22	9.198	<0.001
Progressive motility (%)	25.74±11.55	23.76 ± 13.68	25.41 ± 12.68	28.88 ± 11.13	10.107	<0.001
Normal morphology (%)	1.57 ± 0.62	1.5 ± 0.60	1.7 ± 0.78	1.8 ± 0.85	11.747	<0.001

*The age range of subjects was 20 to 55 years

5.4.1 Attributes of the Study Samples

Semen samples from 1,345 male patient reports were examined in total. The average volume, sperm count, total motility, progressive motility, and normal morphology of all samples assessed were (2.0 ± 0.8) mL, (48.7 ± 21.2) million/mL, (44.1 ± 15.6) %, (25.7 ± 12.6) %, and (1.57 ± 0.62), respectively.

The proportion of patients with normal semen volume (≥1.5 mL), normal sperm count (≥15 × 106/mL), and normal total motility (≥40%) was 81.6, 91.4, and 44.7%, respectively.

5.4.2 Seasonal Variation in Semen Parameters

For comparing the average values of the semen parameters according to the four seasons, we performed an ANOVA test. No statistically significant variation was observed in the mean volume between seasons ($p > 0.05$), whereas count, total motility, progressive motility, and morphology showed a significant variation during different seasons ($p < 0.01$).

In Figure 48, each data point represented as a dot on the graph. the density of the data points, which is the normal morphology per patient, is correlated with the width of the violin at the location. in semen parameters from monsoon to winter, depicting recovery in sperm count, total motility, and progressive motility. corresponds to the total motility values per patient. The colors green, orange, blue, and pink represent patients in summer, monsoon, autumn, and winter, respectively. The V-shaped structure and crowded dot structure in winter in the range of 40–60% motility depict a greater number

of patients lying within the range of 40–60% for total motility. Whereas a greater number of patients lay in the range of 0–40% during the monsoon season compared to other seasons. Summer had a decreasing frequency from 40 to 60% and had the maximum number of patients in the range of around 40%.

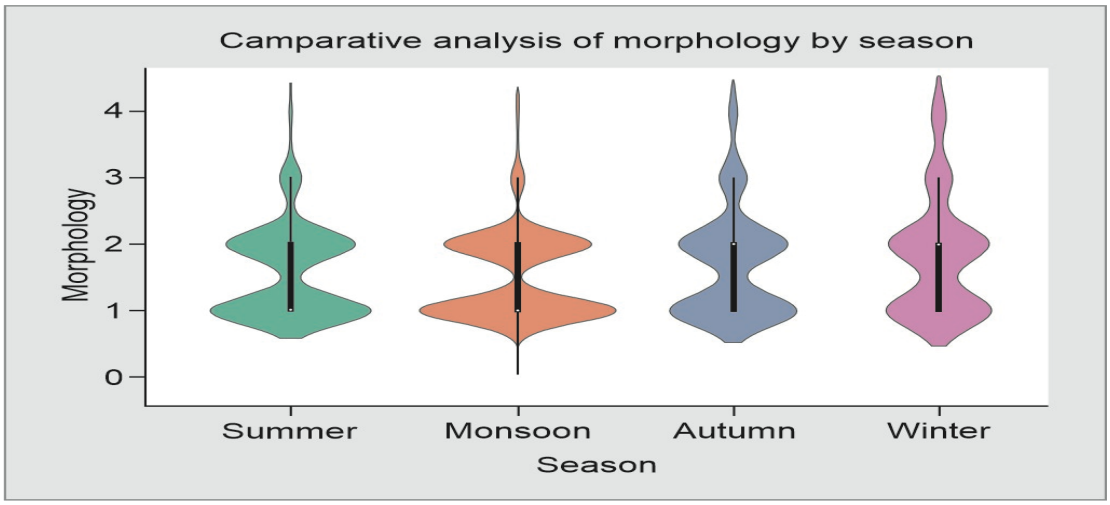


Figure 48: Violin plot representing normal morphology for different seasons

In Figure 49, each data point represented as a dot on the graph is a value of the progressive motility per patient. Green, orange, blue, and pink color is for patients in summer, monsoon, autumn, and winter, respectively. The V-shaped structure and crowded dot structure in winter in the range of 20–40% motility depict more patients lying in the range of 20–40% for progressive motility.

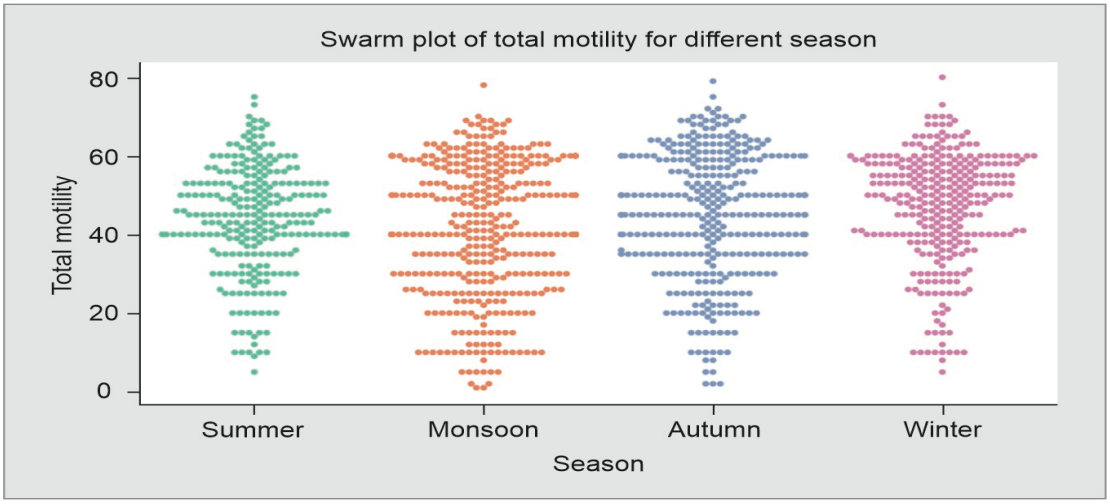


Figure 49: Swarm plot representing total motility for different seasons

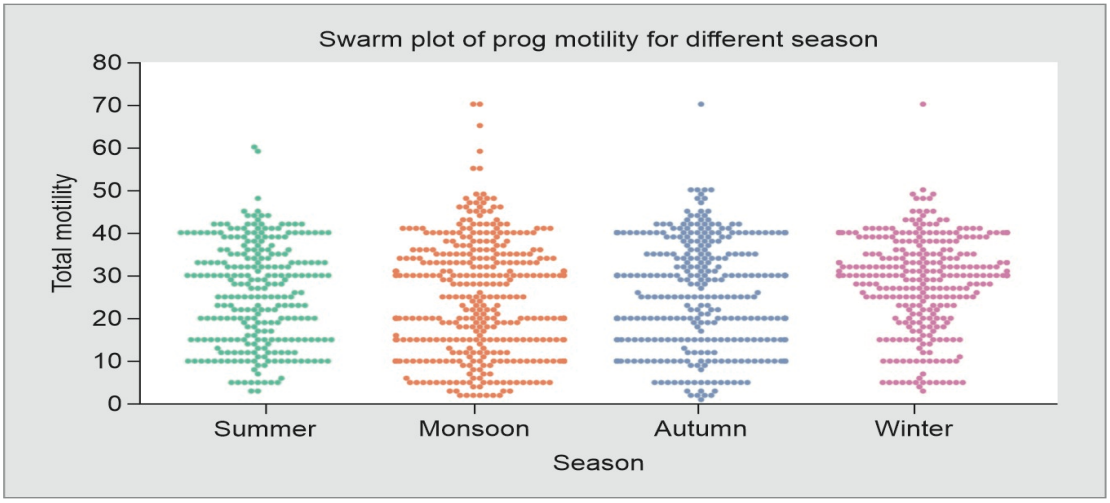


Figure 50: Swarm plot representing progressive motility for different seasons

Where more patients lie in the range of 0–20% in the monsoon season, comparatively. A comparatively broader violin in summer and monsoon than in other seasons appears to be seen between 1 and 2% normal morphology. A broader violin in autumn and winter compared to other seasons appears to be seen between 3 and 4% normal morphology. Box plot: The edges of the box inside the violin represent the interquartile range, and the whiskers show the diversity beyond the higher and lower quartiles

5.5 Discussion

The study analysed semen parameters to investigate the influence of various risk factors on functional aspects such as volume, sperm count, total motility, progressive motility, and morphology, as well as molecular aspects like the DFI. These parameters are examined to assess the quality of semen associated with male infertility. The obtained results were analysed using p-values and correlation coefficients, as shown in the previous section. Furthermore, we will discuss the overall impact of these factors on semen parameters and DFI to investigate their influence on semen quality.

5.5.1 Different Factors vs Functional/Molecular parameters

Different health factors like age, BMI, previous medical history, and stress are considered as health factors to analyse the impact on semen quality.

5.5.1.1 Age

According to the results, there is a gradual increase in semen parameters, such as sperm count, as well as DFI values with increasing age, from the younger age groups (<25 years) to the middle age groups (31-35 years), with a slight decrease in motility in the older age groups (>45 years). However, the differences in DFI values between age groups are not substantial. The statistical analysis reveals correlation coefficient values approaching 1, signifying a robust positive linear correlation between age and semen parameters. This suggests that there is a tendency for semen parameters to increase with age. The p-values demonstrate that there is no significant disparity in semen parameters between the age groups. Although some semen parameters, like sperm count, TM, PR, and DFI, show slight variations across different age groups, these differences are relatively small. However, age groups above 30 exhibit DFI values above the normal range indicating poor sperm quality. This suggests that while age may contribute to some changes in semen quality, there are likely other contributing factors involved. Hence, the findings suggest that age may have a modest impact on semen quality. Age and semen quality must be studied with bigger samples and other parameters to completely understand the link.

5.5.1.2 BMI

According to the results, the impact of BMI on semen quality is observed based on semen parameters and DFI values. The normal weight group (18.5-24.9) shows higher values for sperm count, TM, PR, and morphology compared to other groups. The underweight group (<18.5) and overweight group (25-29.9) show a slight decrease in semen parameters, while the obese group (≥30) exhibits lower values. As shown by the p-values, semen parameters change significant ly between underweight and overweight BMI groups. DFI values of the normal weight group (25.99%) and overweight group (30.88%) are relatively lower, indicating better semen quality compared to the underweight group (33.56%) and obese group (50.06%). Thus, the results highlight the adverse impact of abnormal BMI on semen quality. The correlation coefficient shows a strong negative correlation among BMI and semen parameters, indicating that as BMI increases, semen quality tend to decrease. Therefore, the study suggests that BMI have a modest impact on semen quality. The overweight and normal weight groups tend to

have better semen quality in terms of DFI values compared to the underweight and obese groups. More study with larger sample numbers and more aspects is needed for an accurate understanding BMI and semen quality.

5.5.1.3 Medical History

The study found that previous medical history has no significant impact on semen parameters, as there is a slight increase in semen parameters like sperm count and morphology in the group with previous medical history. The p-value indicates that the statistical difference between the groups with and without medical history is not significant. However, the correlation coefficient shows a linear relationship between the semen parameters analysed in both groups. DFI values also show a slight decrease in the group with medical history compared to the group without medical history. Furthermore, both groups have DFI values higher than the significant threshold, indicating the presence of abnormal sperm. Overall, the study indicates that there is no significant impact of medical history on semen quality.

5.5.1.4 Impact of Lifestyle Factors on semen

Lifestyle factors like smoking, drinking, and smoking affect semen quality, affecting parameters and DFI values. Also, the consumption level of these addictions is observed to impact semen quality.

1. Cigarette smoking

The results reveal a decrease in semen parameters in the smoker group compared to the non-smoker group. Additionally, when comparing different levels of smoking consumption, lower consumption groups (1-2, 3-4) show a decrease in semen parameters, while higher consumption group (>5) shows an increase. This suggests a variation in semen parameters in relation to smoking. Cigarette smoking significant ly affects semen parameters (p-values <0.05). Furthermore, the strong positive linear relationship indicated by the correlation coefficient of 0.99 highlights a consistent and direct association between cigarette smoking frequency and the observed changes in the parameters. This high correlation coefficient suggests that as the frequency of cigarette smoking increases, the semen parameters are consistently affected. DFI values in both smoker and non-smoker groups demonstrate a significant difference, emphasizing the negative impact of cigarette smoking on semen health. Moreover, both

groups exhibit higher DFI values, indicating abnormal semen quality. Additionally, DFI values increase with an increase in smoking frequency, with DFI was highest in those smoking more than five cigarettes each day. This indicates a greater impact of smoking on semen quality and its association with reproductive health in men. Overall, Cigarette smoking degrades semen, harming men's health.

2. Tobacco consumption

The results show an inverse relationship between tobacco consumption and sperm count, as well as total motility. The p-values for the groups consuming 1-3 and 3-6 tobacco per day suggest a significant impact of tobacco consumption on semen parameters. However, in the group consuming more than 6 tobacco per day, the higher p-value indicates a lack of statistical significance as in the observed differences. The high correlation coefficients indicate a consistent and direct association between the quantity of tobacco consumed and semen parameters. The slightly higher DFI in the tobacco-consuming group indicates a negative impact of tobacco consumption on semen quality. Both groups, however, exhibit DFI values higher than the significant level, indicating the presence of abnormal sperm. The group consuming more than 6 tobacco per day shows a decline in DFI, suggesting a potential positive impact of tobacco consumption. Overall, the findings suggest a positive impact of tobacco consumption on semen quality. However, tobacco consumption has detrimental effects on overall health and is associated with numerous other negative health outcomes.

3. Alcohol consumption

The results obtained higher values of semen parameters in the alcohol consumption group. While the higher p-value suggests no statistically significant in the observed differences in semen parameters between the alcohol-consuming and non-consuming groups. However, the high correlation coefficient suggests a strong positive linear relationship between alcohol consumption and the analysed semen parameters. The slight increase in DFI values in the alcohol-consuming group, suggests a negative impact of alcohol on semen quality. However, both groups exhibited higher DFI values indicating the presence of abnormal sperm. Thus, alcohol use significant ly affects semen quality.

Furthermore, Different levels of alcohol consumption within the specified groups have varying impacts on semen parameters. Semen parameters and DFI are increased in the group consuming a high frequency of alcohol indicating no correlation between alcohol consumption and semen parameters.

Therefore, the overall impact of alcohol consumption on semen quality is negative while alcohol frequency shows a positive impact on semen quality. However, the relatively small number of participants in some groups suggests that more research is needed to determine how alcohol use affects semen quality.

5.5.1.5 Vegetarian and Non-vegetarian Diet

The results suggest that following a vegan diet may have a positive impact on semen parameters compared to a non-vegan diet, as the vegan group demonstrates better semen quality. The p-value of 0.0506 indicates a statistically significant impact of the vegan diet compared to the non-vegan diet, while the correlation coefficient suggests a consistent and direct association between the diet and the observed changes in semen parameters. Additionally, individuals following a non-vegetarian diet have slightly higher DFI values, potentially indicating poorer semen quality compared to those following a vegetarian diet. However, the difference in DFI is relatively small, and DFI is above the normal range in both groups. Therefore, diet may have some influence on semen quality.

5.5.1.6 Use of Filtered Water and Non-filtered Water

The analysis reveals an increase in semen parameters in the group using a water purifier, indicating a significant impact of filtered water on semen quality. Additionally, the p-value of 0.0651 suggests a potential but inconclusive effect of filtered water on semen parameters, and the correlation coefficient implies a consistent and direct association between consuming filtered water and altered semen parameters. Although the group using water filters shows a slightly higher DFI compared to the non-filter group, both groups have DFI values exceeding 30%, indicating the presence of abnormal sperm. Since the observed difference between the two groups is relatively small it is not significant. Therefore, the use of water filters does not have a significant impact on DFI values or overall semen quality. However, further research is necessary to establish a

more definitive understanding of the relationship between filtered water consumption and semen parameters.

5.5.1.7 Fast Food

Fast meals may harm semen parameters, resulting in lower values and increased combined defects. The observed changes in semen parameters between the two groups indicate a potential impact of fast-food consumption on male reproductive health, supported by a statistically significant p-value. The correlation coefficient of 0.9997 suggests a strong positive linear relationship between fast food consumption and the analysed semen parameters, indicating the negative impact of fast food on semen quality. Additionally, the fast food-consuming group exhibits slightly higher DFI values compared to the non-consuming group, indicating the potential negative effect on semen quality. Overall, fast food consumption lowers semen quality. However, a full investigation is needed to determine how fast eating affects semen quality.

5.5.1.8 Food Supplement

The results show an increase in semen parameters in the non-consuming group compared to the consuming group of food supplements. These findings suggest that food supplements may affect semen parameters and quality. Furthermore, the p-value suggests a statistically significant difference in semen parameters, while the correlation coefficient implies a consistent and direct association between the consumption of food supplements and semen parameters. The group consuming food supplements exhibits higher DFI values, indicating a higher presence of abnormal sperm. Overall, these results indicate a potential association between the consumption of food supplements and increased DFI values, suggesting a negative impact on semen quality.

5.5.1.9 Tea/coffee consumption

The results reveal minimal differences in semen health among the tea/coffee-consuming and non-consuming groups. The semen parameters in the tea/coffee-consuming group show slight increases, indicating a minimal impact on semen parameters. The p-value suggests a potential impact on semen parameters, while the correlation coefficient implies a consistent association between tea/coffee consumption

and semen parameters. The lower tea/coffee consumption groups (1-2, 3-4) exhibit higher values of semen parameters, but there is a decline in the higher tea/coffee-consuming group (>5), indicating no significant impact of tea/coffee on semen parameters. The p-values suggest a positive impact in the low-consumption groups, while there is no statistically significant impact on semen parameters in the higher tea/coffee-consuming (>5) groups. The correlation coefficients indicate a strong positive linear relationship between tea/coffee intake and semen parameters. Therefore, the impact of tea/coffee intake on semen parameters may vary depending on the level of consumption. There is no significant difference in DFI for the group consuming tea/coffee compared to the non-consuming group, indicating no significant impact on semen quality. However, the higher DFI percentage in both groups indicates the presence of abnormal sperm. An increasing level of tea/coffee consumption may have an impact on DFI values and potentially on semen quality, suggesting a higher presence of abnormal sperm. However, the differences in DFI values between the groups are relatively small. Overall, tea/coffee consumption has no significant impact on semen quality.

5.5.1.10 Carbonated Drink consumption

Cold drink consumption shows a significant decline in semen parameters. The p-value suggests a statistically significant difference in semen parameters, while the correlation coefficient shows a consistent association between the two groups, indicating the negative effect of cold drink consumption on semen parameters. Furthermore, the significant difference in DFI values between the groups consuming cold drinks and those who do not, indicating higher DFI in the group consuming cold drinks, suggests a potential negative impact of cold drink consumption on semen quality. Higher DFIs indicate defective sperm. However, the difference in DFI values and standard deviation between the two groups is relatively large, indicating a greater variation in DFI values within the group. Hence, the consumption of cold drinks may have a negative impact on DFI values and potentially on semen quality. Overall, cold drink consumption shows a negative impact on semen quality.

5.5.1.11. Impact of Caffeine intake (tea/coffee and Cold Drink)

The intake of caffeine, including tea/coffee and cold drinks, shows higher semen parameters, while the group not consuming caffeine showed slightly higher semen volume and morphology. However, the effect of caffeine on parameters of sperm was not statistically noteworthy. The p-value suggests no statistically significant relationship between caffeine intake and semen parameters, as the observed differences in semen parameters could potentially be due to chance alone. Furthermore, the correlation coefficient indicates a strong positive linear relationship between caffeine intake and semen parameters. Moreover, a significant difference in DFI values between the two groups, with higher DFI in the caffeine-consuming group, indicates that Caffeine lowers the quality of semen, showing a higher presence of abnormal sperm. The difference in DFI further emphasizes the impact of caffeine intake on DFI. Therefore, caffeine consumption may contribute to increased DNA fragmentation in sperm, indicating potential negative effects on semen quality. Overall, Caffeine could influence semen quality, but additional investigation needs to be done.

After analysing the dietary factors, we appear to conclude that using filtered water and consuming tea/coffee has no significant impact on semen quality. However, the type of diet, including a vegan diet, may have some influence on semen quality. On the other hand, certain dietary factors such as caffeine intake, fast food consumption, consumption of food supplements, and cold drink consumption show a negative impact on semen quality. It is important to note that further in-depth studies with larger sample sizes may be necessary to establish a stronger correlation between diet and semen quality.

5.5.1.12 Pesticides

Results showed that pesticide-exposed individuals had slightly greater parameters for semen than non-exposed men. These data imply that pesticide exposure may improve semen parameters, stressing the need for more study on pesticides and semen quality. The p-value indicates a statistically significant difference in semen parameters between the group exposed to pesticides and the non-exposed group, further supporting the impact of pesticide exposure on semen parameters. Furthermore, the correlation

coefficient indicates a strong positive linear relationship between pesticide exposure and semen parameters. Thus, pesticide exposure may have a significant impact on semen parameters. Furthermore, the results show a slightly higher DFI in the group without pesticide exposure compared to the group with pesticide exposure. However, both groups exhibit DFI values above the significant threshold (30%), indicating the presence of abnormal sperm in both cases. A small variation in DFI values between both groups could not be considered statistically significant. Pesticides affect semen quality, which may affect male reproductive health. To fully grasp this link, more research is needed.

5.5.1.13. Chemicals

The group exposed to chemicals shows slightly higher values for semen parameters, while parameters like PR and volume are not significantly affected compared to the group not exposed to chemicals. However, these differences are relatively small, suggesting a minimal impact of chemicals on semen parameters. While the group exposed to chemicals exhibited slightly higher values for some semen parameters, the differences are not substantial. Additional study is needed to determine how chemicals affect semen quality. The slightly higher p-value suggests no statistically significant difference in semen parameters indicating a trend towards a difference. Additionally, the correlation coefficient indicates a significant positive linear association between chemical exposure and characteristics of semen, suggesting a consistent association between the two. In conclusion, the results do not provide strong evidence for the significant impact of exposure to chemicals on semen parameters. However, the group exposed to chemicals shows a lower DFI compared to the group not exposed to chemicals, with a notable difference in DFI values. The lower DFI values in the group with chemical exposure suggest a potential positive impact of chemicals on semen quality. However, both groups still exhibit DFI values above the significant threshold (30%), indicating the presence of abnormal sperm in both cases. Therefore, while there is a significant difference in DFI values between the two groups, extra research and analysis are desired to fully understand the potential effects on DFI and overall semen quality. Thus, the overall impact of chemicals on semen quality is not statistically significant.

5.5.1.14 Combine impact of Pesticides and Chemicals

The results indicate that the group exposed to chemicals and pesticides exhibits slightly higher values for semen parameters compared to the non-exposed group. Additionally, the value of the combined defect is slightly lower in the exposed group. It shows a potential positive impact of exposure to chemicals and pesticides on semen parameters, indicating potential improvements in semen quality. However, the observed effects are relatively small. Semen parameters differed significantly comparing exposed and non-exposed categories, according to the p-value. A perfect positive linear association with chemical and pesticide exposure and semen parameters suggests a proportional rise in semen parameters with exposure. Thus, the association between chemicals, pesticides, and semen parameters has a notable impact. Furthermore, the group with exposure to chemicals and pesticides shows a lower DFI compared to the group without such exposure, indicating a potential positive impact of exposure on semen quality. However, both groups still exhibit DFI values above the significant threshold, indicating malformed sperm. In conclusion, exposure to chemicals and pesticides may have a modest impact on DFI and semen quality. Therefore, more study with a greater number of participants along with further factors, such as specific types and concentrations of chemicals and pesticides, is necessary to better understand their effects on semen quality.

5.5.2 Sexual abstinence period vs semen quality

The impact of intercourse frequency on semen parameters was observed. The group with no intercourse per 15 days showed higher semen parameters compared to other groups. However, the groups with higher intercourse frequencies showed declined values of semen parameters. The results suggest that semen parameters are influenced by increasing frequency, but further correlation needs to be investigated. The p-values for higher frequencies of intercourse (more than 10) show significant changes in semen parameters, while lower frequencies (1-5 and 6-10) do not exhibit statistically significant differences but may still demonstrate noticeable trends. Overall, the frequency of intercourse appears to influence semen parameters. The correlation coefficients suggest a strong positive linear relationship, indicating a consistent association with semen parameters and an upsurge in the frequency of intercourse.

However, it does not imply a direct connection and additional study is needed to establish a specific relationship between them. Furthermore, the higher frequency groups (1-10 and 10-20) have a slightly higher DFI than the lower frequency groups (0 and greater than 20). However, the differences in DFI values between the groups are relatively small. Some groups (0 and > 20) exhibit DFI values below the significant threshold (30%), indicating no significant presence of abnormal sperm in these groups. Thus, sexual frequency may have a minor influence on DFI values. Further research is needed to identify other factors causing sperm DNA fragmentation. Overall, sex frequency may affect the quality of semen.

The impact of SAP on semen parameters appears to vary depending on the specific context and underlying factors. According to the results, higher SAP values, particularly in the range of 10-20, may have a positive impact on semen quality. However, SAP values exceeding 20 show a decline in semen parameters, indicating a potential negative impact. The p-values indicate that there is no statistically significant difference in semen parameters among all groups. However, there may still be some variations or trends worth considering. The correlation coefficients suggest a strong positive linear relationship, indicating a consistent association between increasing SAP values and changes in semen parameters. But additional study must be conducted to determine their relationship. The varying SAP levels have no meaningful effect on DFI results. SAP levels appear to have little effect on DFI results. However, Interesting to take into consideration all the groups show DFI values above the significant threshold, suggesting the presence of abnormal sperm regardless of SAP level. Overall, SAP does not have a significant impact on semen quality and requires further investigation and understanding.

Based on the analysis of the results, it appears to be concluded that sexual frequency may have a minor influence on semen quality. However, SAP appears to have little effect on the quality of semen. In summary, sexual factors may play a role in influencing semen quality, but additional studies are required to gain a comprehensive understanding.

5.5.3 Seasonal effects on semen quality

In this study, the winter season had better semen parameters compared to other seasons, whereas monsoon had the least outcomes of semen parameters. The volume of the semen sample showed no significant seasonal variation.

On comparing the mean of count, total motility, progressive motility, and morphology with the yearly mean seasonal temperature, there was no significant correlation, but there was a significant difference when the parameters were correlated only with the seasons. Environmental elements like pesticides and chemicals may not affect semen quality statistically, but seasons may. Research is needed to prove the above factors' impact.

5.6 Summary of results

The present work analysed both functional aspects of semen parameters and molecular aspects like DFI. The examination of different factors revealed their effect on semen quality. The data indicates that health parameters, including age and BMI, exert moderately adverse impacts on the quality of sperm, while past medical treatment and stress appears to additionally have a role. Among lifestyle factors, the tobacco, cigarette smoking negatively impacting both the aspects of on semen quality however, alcohol does not significantly affect semen parameters. Regarding dietary factors, the use of filtered water and consumption of tea/coffee do not have a significant influence on semen quality, while the type of diet, including a veg diet, may have some influence on it. On the other hand, certain dietary factors like consumption of caffeine, fast food, food supplements, and cold drink exhibit a negative impact on semen quality. The sexual frequency may have a minor influence on semen quality, while SAP does not significantly affect it. Thus, sexual attributes may affect semen quality, but more research is needed. The effect of environmental factors such as pesticides and chemicals may not be statistically significant and necessitates further research. Seasons showing the impact on semen health. However, there is a need for further research with a larger sample size for a better understanding of the seasonal impact on semen quality.

5.7 Conclusion

Infertility is a big issue that affects many couples globally, making diagnosis and treatment difficult because it is a couple's complaint rather than an individual problem. Male infertility is on the rise globally, accounting for a sizable number of infertility problems. Male infertility is frequently caused by abnormal spermatogenesis or spermiogenesis. Stress, sleep quality, physical activity, and food appears to all have impact on sperm parameters in infertile males, highlighting the importance of lifestyle in determining fertility. Semen quality, which is impacted by environmental, genetic, and lifestyle variables, is critical to male fertility.

Because to the elevated prevalence of male infertility and the influence of numerous variables on sperm quality, such as health, lifestyle, diet, sexual function, and environmental factors, there is an urgent need for comprehensive studies examining the complex interactions between these risk factors and male infertility. The objective of the current investigation is to assess the impact of these risk variables on both functional and molecular aspects of sperm quality in male infertility, with the goal of finding prospective targets for intervention and prevention.

Significant changes in semen parameters including variations in DFI values were identified in the study, indicating that the investigated factors influenced semen quality. A link between these parameters and sperm quality was discovered through meticulous data analysis. To assess the overall impact on semen quality, the analysis investigated numerous aspects, including their separate sub-elements, in connection to semen parameters and DFI. Based on the results, the study concluded which factors have a substantial impact and which factors do not have a substantial impact on sperm quality.

The research investigates the effects of diet, lifestyle, environment, seasons, and health on male reproductive health, specifically the quality of sperm. To determine the risk factors linked with male infertility, sperm analysis is performed on patients experiencing male infertility and seeking treatment. Individuals of various ages and with a variety of diet and lifestyle habits are involved in the investigation to assess the influence of all these variables on sperm quality related with male infertility. This study

looked at the influence of various factors on the functional aspects of sperm parameters like volume, sperm count, total motility, progressive motility, morphology, and combined defects, as well as molecular aspects like DFI for male infertility. The SCD test applied to analyse sperm parameters and DNA fragmentation measure the impact of risk factors like health, lifestyle, diet, sexual function, as well as environmental factors on sperm quality.

The results of this investigation offer essential insights about the complicated relationships between these risk variables and sperm quality. Tobacco, cigarette smoking, alcohol use, caffeine intake, fast food consumption, and cold drink consumption were observed to have a poor impact on sperm quality. While, age, BMI, medical history, and stress shown a moderately detrimental impact on sperm quality. Though, the type of food, including a vegan diet, and specific dietary characteristics such as drinking filtered water and drinking tea/coffee were identified as potential influencers of sperm quality. Furthermore, sexual frequency was found to have a slight effect on sperm quality, whereas sexual abstinence duration had no effect. The study revealed that environmental factors, particularly pesticides and chemicals, did not demonstrate statistical significance; nonetheless, seasonal impacts were observed, indicating a need for additional investigation to elucidate their potential impact on sperm quality.

Also, these findings illustrate important information to the management of infertility among men. Using this information, healthcare providers appear to design focused interventions and strategies to enhance men reproductive outcomes by evaluating the positive and negative influence of several risk factors on sperm quality. Risk factors like Age, BMI, medical history, and stress have a moderate negative impact on sperm quality that appears to be addressed through appropriate medical interventions, stress management techniques, and lifestyle changes, to improve sperm quality increasing the chances of successful conception. The sperm quality improvement and overall reproductive health comprises behaviour changes and quitting habits in case of lifestyle factors for example tobacco and cigarette smoking, consumption of alcohol, caffeine, fast food, and cold drink.

The study emphasises the possible advantages of certain dietary factors such as eating a vegan diet, drinking filtered water, and drinking tea/coffee indicating that a healthier diet and lifestyle appears to recover sperm quality as well as reproductive potential. The study found that sexual frequency has a minimal impact on sperm quality. However, the sexual abstinence period had no significant effect, displaying that minor fluctuations in sexual activity may not have a substantial impact on sperm quality. Also, environmental factors appear to have a substantial impact on reproductive health indicating need for more research to fully comprehend the impact of environmental variables on the health of sperm.

As a result, the findings deliver important insights into the positive and negative effects of risk factors on sperm quality in male infertility. These are helpful for healthcare practitioners to build personalised treatment plans, with implementation of lifestyle changes, and techniques for enhancing sperm quality and augmenting satisfactory reproductive outcomes.

The study also emphasises the significance as of limitations like limited generalizability, potential confounding factors, retrospective design, and variability in assessment procedures. They highlight the necessity of further research with prospective study designs and larger sample sizes, with additional confounding factors, improved techniques, and intervention studies to establish causal relationships. This study enhances complicated interactions between risk factors and sperm quality in male infertility. Also, the findings emphasise the need of considering several criteria when assessing and treating male infertility. Further study is essential for increasing effectiveness, optimise interventions, as well as develop focused techniques to recover sperm quality and, finally, address the issues faced by male infertile couples.

5.8 Future Scope

Male infertility is acknowledged as a challenging problem affecting millions of couples around the world. As a result, male infertility is a global concern, and understanding the factors and their negative impact on sperm quality is crucial to develop effective interventions for improving male reproductive health. The study addressed many risk factors, such as age, BMI, and lifestyle, which appears to alter the functional and molecular aspects of sperm quality in infertile guys. The study also looked at the underlying mechanisms and pathways that these risk variables affect, as well as the importance of these factors in male infertility. The work highlights multiple subjects for future investigation that may enhance our comprehension of the influence of variables on sperm quality.

A bigger sample size could be added to the study to increase statistical power and generalizability of the findings. A varied range of participants from various populations and areas would aid in capturing potential variations in the impact of risk variables on sperm quality. Using a prospective study design using real-time data appears to help to reduce recollection bias. Other complicating factors influencing sperm quality, such as genetic factors or specific medical disorders, might help to a more complete knowledge of the intricate relationships between risk factors and sperm quality.

Measurement techniques that are standardised and trustworthy appears to improve the accuracy and consistency of data across diverse study settings. Intervention studies involving particular risk variables would aid in establishing causal links and determining the usefulness of interventions in enhancing sperm quality. Assessing the influence of variables on sperm quality across multiple demographics, cultures, and geographical locations may clarify the significance as of contextual factors. The interdisciplinary approach may result in a more complete understanding of the various elements impacting sperm quality. By addressing these future research topics, the project will be able to increase understanding on the influence of factors on sperm quality and develop focused interventions and preventive techniques to reduce the burden of male infertility.

References

1. Suchocka, L., et al., *Social and psychological support of couples in treating infertility (case study of the Polish Research).* Ekonomicheskie i Sotsialnye Peremeny, 2021. **14**(2): p. 188-200.
2. Wal, A., et al., *Conventional treatment options and herbal remedies for male infertility: An overview.* Asian Pacific Journal of Reproduction, 2022. **11**(4): p. 158-164.
3. O'meara, C., et al., *The effect of adjusting settings within a Computer-Assisted Sperm Analysis (CASA) system on bovine sperm motility and morphology results.* Animal Reproduction, 2022. **19**(1): p. e20210077.
4. Zańko, A., et al., *Lifestyle, insulin resistance and semen quality as co-dependent factors of male infertility.* International journal of environmental research and public health, 2022. **20**(1): p. 732.
5. Pozzi, E., R. Ramasamy, and A. Salonia, *Initial andrological evaluation of the infertile male.* European urology focus, 2023. **9**(1): p. 51-54.
6. Dugule, D.S.-G., E.D. Gana, and J. Paul, *Causes, Effects and Management of Infertility Among Reproductive Women in Karim Lamido Local Government Area of Taraba State-Nigeria.* Texas Journal of Medical Science, 2023. **16**: p. 36-49.
7. Schumacher, A.E., et al., *Global fertility in 204 countries and territories, 1950–2021, with forecasts to 2100: a comprehensive demographic analysis for the Global Burden of Disease Study 2021.* The lancet, 2024.
8. EDITION, F., *KNOBIL AND NEILL'S PHYSIOLOGY OF REPRODUCTION.* 2015.
9. Okonofua, F.E., et al., *Causes and risk factors for male infertility: a scoping review of published studies.* International Journal of General Medicine, 2022: p. 5985-5997.
10. Krausz, C., et al., *Genetics of male infertility.* Emery and rimoin's principles and practice of medical genetics and genomics, 2022: p. 121-147.
11. Tüttelmann, F., C. Ruckert, and A. Röpke, *Disorders of spermatogenesis: perspectives for novel genetic diagnostics after 20 years of unchanged routine.* Medizinische Genetik, 2018. **30**(1): p. 12-20.
12. Kuroda, S., et al., *Genetic disorders and male infertility.* Reproductive Medicine and Biology, 2020. **19**(4): p. 314-322.
13. López-Botella, A., et al., *Impact of heavy metals on human male fertility—An overview.* Antioxidants, 2021. **10**(9): p. 1473.
14. Zhang, Y., S. Li, and S. Li, *Relationship between cadmium content in semen and male infertility: a meta-analysis.* Environmental science and pollution research, 2019. **26**: p. 1947-1953.
15. Emokpae, M.A. and S.I. Brown, *Effects of lifestyle factors on fertility: practical recommendations for modification.* Reproduction and Fertility, 2021. **2**(1): p. R13-R26.
16. Agarwal, A., K. Leisegang, and P. Sengupta, *Oxidative stress in pathologies of male reproductive disorders*, in *Pathology*. 2020, Elsevier. p. 15-27.
17. Ranganathan, P., K.A. Rao, and S. Thalaivarasai Balasundaram, *Deterioration of semen quality and sperm-DNA integrity as influenced by cigarette smoking in fertile and infertile human male smokers—A prospective study.* Journal of cellular biochemistry, 2019. **120**(7): p. 11784-11793.
18. Pan, D., et al., *Effects of bisphenol A exposure on DNA integrity and protamination of mouse spermatozoa.* Andrology, 2020. **8**(2): p. 486-496.
19. Esteves, S.C., D. Santi, and M. Simoni, *An update on clinical and surgical interventions to reduce sperm DNA fragmentation in infertile men.* Andrology, 2020. **8**(1): p. 53-81.

20. Nateghian, Z. and E. Aliabadi, *Aspects of environmental pollutants on male fertility and sperm parameters.* J. Environ. Treat. Tech, 2020. **8**(1): p. 299-309.
21. Dutta, S., et al., *Oxidative stress, testicular inflammatory pathways, and male reproduction.* International journal of molecular sciences, 2021. **22**(18): p. 10043.
22. Cervi, L. and D. Knights, *Organizing male infertility: Masculinities and fertility treatment.* Gender, Work & Organization, 2022. **29**(4): p. 1113-1131.
23. Lotti, F. and M. Maggi, *Sexual dysfunction and male infertility.* Nature Reviews Urology, 2018. **15**(5): p. 287-307.
24. Iordachescu, D.A., et al., *Emotional disorders, marital adaptation and the moderating role of social support for couples under treatment for infertility.* Ginekologia polska, 2021. **92**(2): p. 98-104.
25. Mustafa, M., et al., *Male and female infertility: causes, and management.* IOSR Journal of Dental and Medical Sciences, 2019. **18**(9): p. 27-32.
26. Deshpande, P.S. and A.S. Gupta, *Causes and prevalence of factors causing infertility in a public health facility.* Journal of human reproductive sciences, 2019. **12**(4): p. 287-293.
27. Niederberger, C., *Re: Aberrant sperm dna methylation predicts male fertility status and embryo quality.* Journal of Urology, 2016. **196**(4): p. 1231.
28. Organization, W.H., *WHO laboratory manual for the examination and processing of human semen*. 2021: World Health Organization.
29. Huggins, C., W.W. Scott, and J.H. Heinen, *Chemical composition of human semen and of the secretions of the prostate and seminal vesicles.* Ameriappears to Journal of Physiology-Legacy Content, 1942. **136**(3): p. 467-473.
30. Gupta, S. and A. Kumar, *The human semen.* Basics of Human Andrology: A Textbook, 2017: p. 163-170.
31. Singh, R. and K. Singh, *Male infertility: understanding, causes and treatment*. 2017: Springer.
32. Javed, A., M.S. Talkad, and M.K. Ramaiah, *Evaluation of sperm DNA fragmentation using multiple methods: a comparison of their predictive power for male infertility.* Clinical and experimental reproductive medicine, 2019. **46**(1): p. 14.
33. Bisconti, M., et al., *Influence of risk factors for male infertility on sperm protein composition.* International Journal of Molecular Sciences, 2021. **22**(23): p. 13164.
34. Barbăroșie, C., A. Agarwal, and R. Henkel, *Diagnostic value of advanced semen analysis in evaluation of male infertility.* Andrologia, 2021. **53**(2): p. e13625.
35. Bracke, A., et al., *A search for molecular mechanisms underlying male idiopathic infertility.* Reproductive biomedicine online, 2018. **36**(3): p. 327-339.
36. e Silva, K.S.F., *Molecular genetics of male infertility: A mini-review.* Trends Res, 2018. **1**(2).
37. Gao, J., et al., *Age-related changes in human conventional semen parameters and sperm chromatin structure assay-defined sperm DNA/chromatin integrity.* Reproductive BioMedicine Online, 2021. **42**(5): p. 973-982.
38. Condorelli, R.A., et al., *Diabetes mellitus and infertility: different pathophysiological effects in type 1 and type 2 on sperm function.* Frontiers in endocrinology, 2018. **9**: p. 268.
39. research, Z.m. *Male infertility Treatment market report on size, survey and trends*. 2021; Available from: http://secure.rezserver.com/sdk/v1/LinkFwd?refid=7684&destination=agoda&refclickid=websliceW1018.

40. Tofighi Niaki, M., et al., *Possible harmful effects of smoking hookah on sperm DNA fragmentation index and protamine genes expression in normozoospermic men.* Substance abuse: research and treatment, 2023. **17**: p. 11782218221144547.
41. Gallo, A., *Reprotoxic impact of environment, diet, and behavior.* International Journal of Environmental Research and Public Health, 2022. **19**(3): p. 1303.
42. Luo, K., et al., *Environmental exposure to legacy poly/perfluoroalkyl substances, emerging alternatives and isomers and semen quality in men: A mixture analysis.* Sci Total Environ, 2022. **833**: p. 155158.
43. Sofikitis, N., et al., *The Role of Pharmaceutical Agents in the Alleviation of Male Infertility (Part-I).* Curr Pharm Des, 2021. **27**(23): p. 2661-2664.
44. Tahmasbpour, E., D. Balasubramanian, and A. Agarwal, *A multi-faceted approach to understanding male infertility: gene mutations, molecular defects and assisted reproductive techniques (ART).* J Assist Reprod Genet, 2014. **31**(9): p. 1115-37.
45. Chen, W., et al., *Epigenetic Effects of Assisted Reproductive Technology in Human Offspring.* bioRxiv, 2019: p. 816157.
46. Parrella, A., et al., *High proportion of immature oocytes in a cohort reduces fertilization, embryo development, pregnancy and live birth rates following ICSI.* Reprod Biomed Online, 2019. **39**(4): p. 580-587.
47. Palumbo, A., et al., *Personalized therapy in multiple myeloma according to patient age and vulnerability: a report of the European Myeloma Network (EMN).* Blood, 2011. **118**(17): p. 4519-4529.
48. Del Pup, L., et al., *Risk of appears tocer after assisted reproduction: a review of the available evidences and guidance to fertility counselors.* Eur Rev Med Pharmacol Sci, 2018. **22**(22): p. 8042-8059.
49. Beigi Harchegani, A., et al., *Hyperviscous Semen Causes Poor Sperm Quality and Male Infertility through Induction of Oxidative Stress.* Curr Urol, 2019. **13**(1): p. 1-6.
50. Nieschlag, E. and E. Vorona, *Doping with anabolic androgenic steroids (AAS): Adverse effects on non-reproductive organs and functions.* Rev Endocr Metab Disord, 2015. **16**(3): p. 199-211.
51. Irigoyen, P., et al., *Mitochondrial metabolism determines the functional status of human sperm and correlates with semen parameters.* Frontiers in Cell and Developmental Biology, 2022. **10**.
52. Tahmasbpour Marzouni, E., et al., *Epigenetic Modifications, A New Approach to Male Infertility Etiology: A Review.* Int J Fertil Steril, 2022. **16**(1): p. 1-9.
53. Agarwal, A., et al., *Diagnostic application of oxidation-reduction potential assay for measurement of oxidative stress: clinical utility in male factor infertility.* Reproductive BioMedicine Online, 2017. **34**(1): p. 48-57.
54. Jungwirth, A., et al., *European Association of Urology Guidelines on Male Infertility: The 2012 Update.* European Urology, 2012. **62**(2): p. 324-332.
55. Uhlén, M., et al., *Proteomics. Tissue-based map of the human proteome.* Science, 2015. **347**(6220): p. 1260419.
56. Yu, N.Y.-L., et al., *Complementing tissue characterization by integrating transcriptome profiling from the Human Protein Atlas and from the FANTOM5 consortium.* Nucleic acids research, 2015. **43**(14): p. 6787-6798.
57. Neto, F.T., et al., *Spermatogenesis in humans and its affecting factors.* Semin Cell Dev Biol, 2016. **59**: p. 10-26.
58. Adamson, G.D., et al., *International Committee for Monitoring Assisted Reproductive Technology: world report on assisted reproductive technology, 2011.* Fertility and Sterility, 2018. **110**(6): p. 1067-1080.

59. Ugwuja, E.I., N.C. Ugwu, and B.N. Ejikeme, *Prevalence of low sperm count and abnormal semen parameters in male partners of women consulting at infertility clinic in Abakaliki, Nigeria.* Afr J Reprod Health, 2008. **12**(1): p. 67-73.
60. Araoye, M.O., *Epidemiology of infertility: social problems of the infertile couples.* West Afriappears to journal of medicine, 2003. **22**(2): p. 190-196.
61. Agarwal, A., et al., *Male infertility.* Lancet, 2021. **397**(10271): p. 319-333.
62. Karavolos, S., et al., *An update on the management of male infertility.* The Obstetrician & Gynaecologist, 2020. **22**(4): p. 267-274.
63. Zhang, G., et al., *Associations of ambient air pollutant exposure with seminal plasma MDA, sperm mtDNA copy number, and mtDNA integrity.* Environment International, 2020. **136**: p. 105483.
64. Sharpe, R.M., *Environmental/lifestyle effects on spermatogenesis.* Philosophical Transactions of the Royal Society B: Biological Sciences, 2010. **365**(1546): p. 1697-1712.
65. Statista. *Total fertility rate in India, from 1880 to 2020*. 2020.
66. www.prsindia.org, *Vital Stats – National Family Health Survey 5*. 2020.
67. Nayana, V. and J. Sandeep, *From Boom to Bust: Unpacking India's Fertility Decline.* Indian Journal of Research in Anthropology, 2024. **10**(1).
68. Mascarenhas, M.N., et al., *National, regional, and global trends in infertility prevalence since 1990: a systematic analysis of 277 health surveys.* PLoS Med, 2012. **9**(12): p. e1001356.
69. Agarwal, A., et al., *A unique view on male infertility around the globe.* Reprod Biol Endocrinol, 2015. **13**: p. 37.
70. Nagamatsu, G. and K. Hayashi, *Stem cells, in vitro gametogenesis and male fertility.* Reproduction, 2017. **154**(6): p. F79-F91.
71. Fang, F., et al., *Human induced pluripotent stem cells and male infertility: an overview of current progress and perspectives.* Human Reproduction, 2018. **33**(2): p. 188-195.
72. Neuhaus, N. and S. Schlatt, *Stem cell–based options to preserve male fertility.* Science, 2019. **363**(6433): p. 1283-1284.
73. Durairajanayagam, D., *Lifestyle causes of male infertility.* Arab journal of urology, 2018. **16**(1): p. 10-20.
74. Minhas, S., et al., *European Association of Urology Guidelines on Male Sexual and Reproductive Health: 2021 Update on Male Infertility.* European Urology, 2021. **80**(5): p. 603-620.
75. Damsgaard, J., et al., *Varicocele Is Associated with Impaired Semen Quality and Reproductive Hormone Levels: A Study of 7035 Healthy Young Men from Six European Countries.* European Urology, 2016. **70**(6): p. 1019-1029.
76. Hussain, A. and J. Gilloteaux, *The human testes: Estrogen and ageing outlooks.* Translational Research in Anatomy, 2020. **20**: p. 100073.
77. Ozturk, S., *Genetic variants underlying spermatogenic arrests in men with non-obstructive azoospermia.* Cell Cycle, 2023. **22**(9): p. 1021-1061.
78. Horns, J.J., et al., *The impact of socioeconomic status on bulk semen parameters, fertility treatment, and fertility outcomes in a cohort of subfertile men.* Fertil Steril, 2023. **120**(1): p. 72-79.
79. Singh, N.P., C.H. Muller, and R.E. Berger, *Effects of age on DNA double-strand breaks and apoptosis in human sperm.* Fertility and Sterility, 2003. **80**(6): p. 1420-1430.
80. Bosco, L., et al., *Apoptosis in human unfertilized oocytes after intracytoplasmic sperm injection.* Fertility and Sterility, 2005. **84**(5): p. 1417-1423.

81. Desai, N., et al., *Free radical theory of aging: implications in male infertility.* Urology, 2010. **75**(1): p. 14-19.
82. Aksu, A.E. and H. Gunes, *Chapter 26 - Effects of aging on extremities*, in *Beauty, Aging, and AntiAging*, İ. Vargel and F.F. Özgür, Editors. 2023, Academic Press. p. 271-287.
83. Puleo, C.M., et al., *Advancing paternal age and simplex autism.* Autism, 2012. **16**(4): p. 367-80.
84. Luo, L., et al., *Aging and the brown Norway rat leydig cell antioxidant defense system.* J Androl, 2006. **27**(2): p. 240-7.
85. Lawrence, L.T. and K.H. Moley, *Epigenetics and assisted reproductive technologies: human imprinting syndromes.* Semin Reprod Med, 2008. **26**(2): p. 143-52.
86. Emokpae, M.A. and S.I. Brown, *Effects of lifestyle factors on fertility: practical recommendations for modification.* Reprod Fertil, 2021. **2**(1): p. R13-r26.
87. Ricci, E., et al., *Semen quality and alcohol intake: a systematic review and meta-analysis.* Reproductive biomedicine online, 2017. **34**(1): p. 38-47.
88. Niederberger, C., *Re: Alcohol and male reproductive health: a cross-sectional study of 8344 healthy men from Europe and the USA.* J Urol, 2015. **193**(4): p. 1329.
89. Gundersen, T.D., et al., *Association Between Use of Marijuana and Male Reproductive Hormones and Semen Quality: A Study Among 1,215 Healthy Young Men.* Ameriappears to Journal of Epidemiology, 2015. **182**(6): p. 473-481.
90. Gaur, D.S., M.S. Talekar, and V.P. Pathak, *Alcohol intake and cigarette smoking: Impact of two major lifestyle factors on male fertility.* Indian Journal of Pathology and Microbiology, 2010. **53**(1): p. 35-40.
91. Zhang, Z.-B., et al., *Male infertility in Northeast China: a cytogenetic study of 135 patients with non-obstructive azoospermia and severe oligozoospermia.* Journal of assisted reproduction and genetics, 2012. **29**: p. 83-87.
92. Martini, A.C., et al., *Effects of alcohol and cigarette consumption on human seminal quality.* Fertil Steril, 2004. **82**(2): p. 374-7.
93. Rehman, R., et al., *Relationship Between Smoking Habit and Sperm Parameters Among Patients Attending an Infertility Clinic.* Front Physiol, 2019. **10**: p. 1356.
94. Antoniassi, M.P., et al., *Analysis of the functional aspects and seminal plasma proteomic profile of sperm from smokers.* BJU Int, 2016. **118**(5): p. 814-822.
95. Pini, T., et al., *Obesity significant ly alters the human sperm proteome, with potential implications for fertility.* J Assist Reprod Genet, 2020. **37**(4): p. 777-787.
96. Chen, X., et al., *Alteration of sperm protein profile induced by cigarette smoking.* Acta Biochim Biophys Sin (Shanghai), 2015. **47**(7): p. 504-15.
97. Aboulmaouahib, S., et al., *Impact of alcohol and cigarette smoking consumption in male fertility potential: Looks at lipid peroxidation, enzymatic antioxidant activities and sperm DNA damage.* Andrologia, 2018. **50**(3).
98. Zhang, J.-P., et al., *Effect of smoking on semen quality of infertile men in Shandong, China.* Asian journal of andrology, 2000. **2**(2): p. 143-146.
99. Cui, X., et al., *Potential effect of smoking on semen quality through DNA damage and the downregulation of Chk1 in sperm.* Mol Med Rep, 2016. **14**(1): p. 753-61.
100. Kumosani, T.A., et al., *The influence of smoking on semen quality, seminal microelements and Ca2+-ATPase activity among infertile and fertile men.* Clin Biochem, 2008. **41**(14-15): p. 1199-203.
101. Ghaffari, M.A. and M. Rostami, *The effect of cigarette smoking on human sperm creatine kinase activity: as an ATP buffering system in sperm.* Int J Fertil Steril, 2013. **6**(4): p. 258-65.

102. El-Melegy, N.T. and M.E. Ali, *Apoptotic markers in semen of infertile men: Association with cigarette smoking.* Int Braz J Urol, 2011. **37**(4): p. 495-506.
103. Ito, C. and K. Toshimori, *Acrosome markers of human sperm.* Anat Sci Int, 2016. **91**(2): p. 128-42.
104. Chaudhury, K., et al., *Acrosin activity as a potential marker for sperm membrane characteristics in unexplained male infertility.* Fertil Steril, 2005. **83**(1): p. 104-9.
105. Yu, B., et al., *Cigarette smoking is associated with human semen quality in synergy with functional NRF2 polymorphisms.* Biol Reprod, 2013. **89**(1): p. 5.
106. Ramlau-Hansen, C.H., et al., *Is smoking a risk factor for decreased semen quality? A cross-sectional analysis.* Hum Reprod, 2007. **22**(1): p. 188-96.
107. Ji, B.-T., et al., *Paternal cigarette smoking and the risk of childhood appears tocer among offspring of nonsmoking mothers.* Journal of the National Appears tocer Institute, 1997. **89**(3): p. 238-243.
108. Waylen, A.L., et al., *Effects of cigarette smoking upon clinical outcomes of assisted reproduction: a meta-analysis.* Hum Reprod Update, 2009. **15**(1): p. 31-44.
109. Rybar, R., et al., *Sperm chromatin integrity in young men with no experiences of infertility and men from idiopathic infertility couples.* Andrologia, 2009. **41**(3): p. 141-9.
110. Bielawski, D.M., et al., *Paternal Alcohol Exposure Affects Sperm Cytosine Methyltransferase Messenger RNA Levels.* Alcoholism: Clinical and Experimental Research, 2002. **26**(3): p. 347-351.
111. Fronczak, C.M., E.D. Kim, and A.B. Barqawi, *The insults of illicit drug use on male fertility.* Journal of andrology, 2012. **33**(4): p. 515-528.
112. du Plessis, S.S., A. Agarwal, and A. Syriac, *Marijuana, phytoappears tonabinoids, the endoappears tonabinoid system, and male fertility.* J Assist Reprod Genet, 2015. **32**(11): p. 1575-88.
113. Samplaski, M.K., et al., *Cocaine Use in the Infertile Male Population: A Marker for Conditions Resulting in Subfertility.* Curr Urol, 2015. **8**(1): p. 38-42.
114. Kanayama, G., J.I. Hudson, and H.G. Pope, Jr., *Illicit anabolic-androgenic steroid use.* Horm Behav, 2010. **58**(1): p. 111-21.
115. Nieschlag, E. and E. Vorona, *MECHANISMS IN ENDOCRINOLOGY: Medical consequences of doping with anabolic androgenic steroids: effects on reproductive functions.* Eur J Endocrinol, 2015. **173**(2): p. R47-58.
116. Colaco, S. and D. Modi, *Consequences of Y chromosome microdeletions beyond male infertility.* J Assist Reprod Genet, 2019. **36**(7): p. 1329-1337.
117. Jensen, T.K., et al., *Body mass index in relation to semen quality and reproductive hormones among 1,558 Danish men.* Fertil Steril, 2004. **82**(4): p. 863-70.
118. Kort, H.I., et al., *Impact of Body Mass Index Values on Sperm Quantity and Quality.* Journal of Andrology, 2006. **27**(3): p. 450-452.
119. Kriegel, T.M., et al., *Identification of diabetes- and obesity-associated proteomic changes in human spermatozoa by difference gel electrophoresis.* Reprod Biomed Online, 2009. **19**(5): p. 660-70.
120. Asha, A., *Impact of age, verified occupation and lifestyle on semen parameters of infertile males in Jaipur: A preliminary study.* International Journal of Health & Allied Sciences, 2014. **3**(4): p. 278-278.
121. Paasch, U., et al., *Identification of Increased Amounts of Eppin Protein Complex Components in Sperm Cells of Diabetic and Obese Individuals by Difference Gel Electrophoresis.* Molecular & cellular proteomics : MCP, 2011. **10**: p. M110.007187.

122. Liu, Y., et al., *Proteomic pattern changes associated with obesity-induced asthenozoospermia.* Andrology, 2015. **3**(2): p. 247-259.
123. Guo, Y., et al., *Proteomics analysis of asthenozoospermia and identification of glucose-6-phosphate isomerase as an important enzyme for sperm motility.* Journal of Proteomics, 2019. **208**: p. 103478.
124. Ferigolo, P.C., et al., *Sperm functional aspects and enriched proteomic pathways of seminal plasma of adult men with obesity.* Andrology, 2019. **7**(3): p. 341-349.
125. Peng, Y., et al., *Proteomic alterations underlie an association with teratozoospermia in obese mice sperm.* Reprod Biol Endocrinol, 2019. **17**(1): p. 82.
126. Carvalho, M.G., et al., *Effects of Obesity and Diabetes on Sperm Cell Proteomics in Rats.* Journal of Proteome Research, 2021. **20**(5): p. 2628-2642.
127. An, T., et al., *Comparative analysis of proteomes between diabetic and normal human sperm: Insights into the effects of diabetes on male reproduction based on the regulation of mitochondria-related proteins.* Mol Reprod Dev, 2018. **85**(1): p. 7-16.
128. Martínez-Heredia, J., et al., *Identification of proteomic differences in asthenozoospermic sperm samples.* Hum Reprod, 2008. **23**(4): p. 783-91.
129. Organization, W.H., *WHO global report on trends in prevalence of tobacco use 2000-2025*. 2020: World Health Organization.
130. Deepinder, F., K. Makker, and A. Agarwal, *Cell phones and male infertility: dissecting the relationship.* Reprod Biomed Online, 2007. **15**(3): p. 266-70.
131. Gorpinchenko, I., et al., *The influence of direct mobile phone radiation on sperm quality.* Cent European J Urol, 2014. **67**(1): p. 65-71.
132. Al-Bayyari, N., *The effect of cell phone usage on semen quality and fertility among Jordanian males.* Middle East Fertility Society Journal, 2017. **22**(3): p. 178-182.
133. Singh, V. and R. Pakhiddey, *Current Scenario on Genetic Basis of Infertility- A Review.* Acta Medica International, 2015. **2**(2): p. 149-154.
134. Research, Z.M. *Male Infertility Treatment Market Size*. 2021; Available from: https://www.zionmarketresearch.com/report/male-infertility-treatment-techniques-market.
135. He, Y., et al., *Heavy metal exposure, oxidative stress and semen quality: Exploring associations and mediation effects in reproductive-aged men.* Chemosphere, 2020. **244**: p. 125498.
136. Joffe, M., et al., *Time To Pregnancy and occupational lead exposure.* Occup Environ Med, 2003. **60**(10): p. 752-8.
137. Fatima, P., et al., *Relationship of blood and semen lead level with semen parameter.* Mymensingh Medical Journal: MMJ, 2010. **19**(3): p. 405-414.
138. Telisman, S., et al., *Semen quality and reproductive endocrine function in relation to biomarkers of lead, cadmium, zinc, and copper in men.* Environmental health perspectives, 2000. **108**(1): p. 45-53.
139. Akinloye, O., et al., *Cadmium toxicity: a possible cause of male infertility in Nigeria.* Reprod Biol, 2006. **6**(1): p. 17-30.
140. Choy, C.M., et al., *Infertility, blood mercury concentrations and dietary seafood consumption: a case–control study.* BJOG: an international journal of obstetrics and gynaecology, 2002. **109**(10): p. 1121-1125.
141. Mínguez-Alarcón, L., et al., *Correlations between different heavy metals in diverse body fluids: studies of human semen quality.* Advances in Urology, 2012. **2012**(1): p. 420893.

142. Lacasaña, M., et al., *Maternal and paternal occupational exposure to agricultural work and the risk of anencephaly.* Occupational and environmental medicine, 2006. **63**(10): p. 649-656.
143. Mocarelli, P., et al., *Paternal concentrations of dioxin and sex ratio of offspring.* The Lancet, 2000. **355**(9218): p. 1858-1863.
144. Anshina, M.B., et al., *The international glossary on infertility and fertility care.* Russian journal of human reproduction, 2019. **25**(1): p. 6-15.
145. A. Nishida, T.S., and I. Ikemoto, , , *"Effect of genital tract infection on male infertility,".* Tokyo Jikeikai Med. J., vol. 108, no. 6, pp. 821–827, 1993, , Accessed: Apr. 25, 2023. **vol. 108**(no. 6): p. pp. 821–827.
146. Boeri, L., et al., *Semen infections in men with primary infertility in the real-life setting.* Fertility and Sterility, 2020. **113**(6): p. 1174-1182.
147. Sharma, R., et al., *Cigarette smoking and semen quality: a new meta-analysis examining the effect of the 2010 World Health Organization laboratory methods for the examination of human semen.* European urology, 2016. **70**(4): p. 635-645.
148. Ricci, E., et al., *Semen quality and alcohol intake: a systematic review and meta-analysis.* Reprod Biomed Online, 2017. **34**(1): p. 38-47.
149. Agarwal, A., et al., *Effect of vaginal lubriappears tots on sperm motility and chromatin integrity: a prospective comparative study.* Fertility and sterility, 2008. **89**(2): p. 375-379.
150. Agbaje, I., et al., *Insulin dependant diabetes mellitus: implications for male reproductive function.* Human Reproduction, 2007. **22**(7): p. 1871-1877.
151. Dong, Y., et al., *Impact of chromosomal translocations on male infertility, semen quality, testicular volume and reproductive hormone levels.* Journal of International Medical Research, 2012. **40**(6): p. 2274-2283.
152. Harris, I.D., et al., *Fertility and the aging male.* Reviews in urology, 2011. **13**(4): p. e184.
153. Huynh, T., R. Mollard, and A. Trounson, *Selected genetic factors associated with male infertility.* Human Reproduction Update, 2002. **8**(2): p. 183-198.
154. Vicdan, A., et al., *Genetic aspects of human male infertility: the frequency of chromosomal abnormalities and Y chromosome microdeletions in severe male factor infertility.* European Journal of Obstetrics & Gynecology and Reproductive Biology, 2004. **117**(1): p. 49-54.
155. Mau-Holzmann, U., *Somatic chromosomal abnormalities in infertile men and women.* Cytogenetic and Genome Research, 2005. **111**(3-4): p. 317-336.
156. Black, L.D., et al., *Compound genetic factors as a cause of male infertility: case report.* Hum Reprod, 2000. **15**(2): p. 449-51.
157. Asada, H., et al., *The Effects of Age and Abnormal Sperm Count on the Nondisjunction of Spermatozoa.* Journal of Assisted Reproduction and Genetics, 2004. **17**: p. 51-59.
158. Wojda, A., et al., *[Importance of cytogenetic analysis in patients with azoospermia or severe oligozoospermia undergoing in vitro fertilization].* Ginekol Pol, 2001. **72**(11): p. 847-53.
159. Molnar, A.M., G.S. Terasaki, and J.K. Amory, *Klinefelter syndrome presenting as behavioral problems in a young adult.* Nat Rev Endocrinol, 2010. **6**(12): p. 707-12.
160. McCallum, T.J., et al., *Fertility in men with cystic fibrosis: an update on current surgical practices and outcomes.* Chest, 2000. **118**(4): p. 1059-62.
161. Chi, F., et al., *DNA methylation status of imprinted H19 and KvDMR1 genes in human placentas after conception using assisted reproductive technology.* Ann Transl Med, 2020. **8**(14): p. 854.

162. Vieweg, M., et al., *Methylation analysis of histone H4K12ac-associated promoters in sperm of healthy donors and subfertile patients.* Clinical Epigenetics, 2015. **7**(1): p. 31.
163. Montjean, D., et al., *Sperm global DNA methylation level: association with semen parameters and genome integrity.* Andrology, 2015. **3**(2): p. 235-240.
164. Houshdaran, S., et al., *Widespread epigenetic abnormalities suggest a broad DNA methylation erasure defect in abnormal human sperm.* PLoS One, 2007. **2**(12): p. e1289.
165. Wu, W., et al., *Idiopathic male infertility is strongly associated with aberrant promoter methylation of methylenetetrahydrofolate reductase (MTHFR).* PLoS One, 2010. **5**(11): p. e13884.
166. Han, F., et al., *Epigenetic Inactivation of SOX30 Is Associated with Male Infertility and Offers a Therapy Target for Non-obstructive Azoospermia.* Mol Ther Nucleic Acids, 2020. **19**: p. 72-83.
167. Sule, R., G. Rivera, and A.V. Gomes, *Western blotting (immunoblotting): history, theory, uses, protocol and problems.* Biotechniques, 2023. **75**(3): p. 99-114.
168. Chiang, C., S. Mahalingam, and J.A. Flaws. *Environmental contaminants affecting fertility and somatic health*. in *Seminars in reproductive medicine*. 2017. Thieme Medical Publishers.
169. Rengaraj, D., W.-S. Kwon, and M.-G. Pang, *Effects of motor vehicle exhaust on male reproductive function and associated proteins.* Journal of proteome research, 2015. **14**(1): p. 22-37.
170. Wang, X., et al., *Gaseous pollutant exposure affects semen quality in central China: a cross-sectional study.* Andrology, 2020. **8**(1): p. 117-124.
171. Rosa, M.D., et al., *Traffic pollutants affect fertility in men.* Human Reproduction, 2003. **18**(5): p. 1055-1061.
172. Calogero, A., et al., *Environmental car exhaust pollution damages human sperm chromatin and DNA.* Journal of endocrinological investigation, 2011. **34**: p. e139-e143.
173. Wdowiak, A., et al., *Air pollution and semen parameters in men seeking fertility treatment for the first time.* International Journal of Occupational Medicine and Environmental Health, 2019. **32**(3): p. 387-399.
174. Wang, L., et al., *Effects of PM2. 5 exposure on reproductive system and its mechanisms.* Chemosphere, 2021. **264**: p. 128436.
175. Chen, Y., et al., *The impact of the fine ambient particle on infertile male's sperm quality.* Urological Science, 2019. **30**(4): p. 177-183.
176. Sklorz, M., et al., *Concentration of oxygenated polycyclic aromatic hydrocarbons and oxygen free radical formation from urban particulate matter.* Journal of Toxicology and Environmental Health, Part A, 2007. **70**(21): p. 1866-1869.
177. Gaspari, L., et al., *Polycyclic aromatic hydrocarbon-DNA adducts in human sperm as a marker of DNA damage and infertility.* Mutation research/genetic toxicology and environmental mutagenesis, 2003. **535**(2): p. 155-160.
178. Pilsner, J.R., et al., *Spermatogenesis disruption by dioxins: Epigenetic reprograming and windows of susceptibility.* Reprod Toxicol, 2017. **69**: p. 221-229.
179. Carlsen, E., et al., *Evidence for decreasing quality of semen during past 50 years.* British medical journal, 1992. **305**(6854): p. 609-613.
180. Ladipo, O.A., *Seminal analysis in fertile and infertile Nigerian men.* J Natl Med Assoc, 1980. **72**(8): p. 785-9.
181. Geoffroy-Siraudin, C., et al., *Decline of semen quality among 10 932 males consulting for couple infertility over a 20-year period in Marseille, France.* Asian J Androl, 2012. **14**(4): p. 584-90.

182. Shamsi, M., R. Kumar, and R. Dada, *Evaluation of nuclear DNA damage in human spermatozoa in men opting for assisted reproduction.* Indian Journal of Medical Research, 2008. **127**(2): p. 115-123.
183. Mehta, R.H., et al., *Prevalences of oligozoospermia and azoospermia in male partners of infertile couples from different parts of India.* Asian J Androl, 2006. **8**(1): p. 89-93.
184. Hernández Uribe, L., et al., *[Frequency and etiology of azoospermia in the study of infertile couples].* Ginecologia y obstetricia de Mexico, 2001. **69**: p. 322-326.
185. Xie, B.-G., W.-J. Zhu, and Y.-H. Huang, *Outcome of conventional IVF and ICSI on sibling oocytes in moderate ligoasthenozoospermia.* Pakistan Journal of Medical Sciences, 2013. **29**(5): p. 1221.
186. Kumar, N. and A.K. Singh, *Trends of male factor infertility, an important cause of infertility: A review of literature.* J Hum Reprod Sci, 2015. **8**(4): p. 191-6.
187. Organisation, W.H., *WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction*. 1999: Cambridge university press.
188. *Fertility of Men and Women Aged 15–49 in the United States: National Survey of Family Growth, 2015–2019*. 2023, https://dx.doi.org/10.15620/cdc:122080: Hyattsville, MD.
189. Sharlip, I.D., et al., *Best practice policies for male infertility.* Fertil Steril, 2002. **77**(5): p. 873-82.
190. Yu, L., et al., *Delayed covering causes the accumulation of motile sperm, leading to overestimation of sperm concentration and motility with a Makler counting chamber.* Asian Journal of Andrology, 2025. **27**(1): p. 59-64.
191. Gevrek, F., et al., *Evaluation of the aquaporin molecules characterization in the sperm cells of men from different aged.* Turkish Journal of Medical Sciences, 2024. **54**(1): p. 204-212.
192. Björndahl, L. and U. Kvist, *Structure of chromatin in spermatozoa.* Adv Exp Med Biol, 2014. **791**: p. 1-11.
193. Agarwal, A., et al., *Sperm DNA Fragmentation: A New Guideline for Clinicians.* World J Mens Health, 2020. **38**(4): p. 412-471.
194. Esteves, S.C., et al., *Sperm DNA fragmentation testing: Summary evidence and clinical practice recommendations.* Andrologia, 2021. **53**(2): p. e13874.
195. Ribas-Maynou, J. and J. Benet, *Single and Double Strand Sperm DNA Damage: Different Reproductive Effects on Male Fertility.* Genes (Basel), 2019. **10**(2).
196. Panner Selvam, M.K., P. Sengupta, and A. Agarwal, *Sperm DNA fragmentation and male infertility.* Genetics of Male Infertility: A Case-Based Guide for Clinicians, 2020: p. 155-172.
197. Baskaran, S., et al., *Tracking research trends and hotspots in sperm DNA fragmentation testing for the evaluation of male infertility: a scientometric analysis.* Reprod Biol Endocrinol, 2019. **17**(1): p. 110.
198. Agarwal, A., S. Prabakaran, and S. Allamaneni, *What an andrologist/urologist should know about free radicals and why.* Urology, 2006. **67**(1): p. 2-8.
199. Esteves, S.C., et al., *Reproductive outcomes of testicular versus ejaculated sperm for intracytoplasmic sperm injection among men with high levels of DNA fragmentation in semen: systematic review and meta-analysis.* Fertil Steril, 2017. **108**(3): p. 456-467.e1.
200. Aitken, R.J., *DNA damage in human spermatozoa; important contributor to mutagenesis in the offspring.* Transl Androl Urol, 2017. **6**(Suppl 4): p. S761-s764.

201. Agarwal, A., et al., *Clinical utility of sperm DNA fragmentation testing: practice recommendations based on clinical scenarios.* Transl Androl Urol, 2016. **5**(6): p. 935-950.
202. Muratori, M., et al., *Sperm DNA Fragmentation: Mechanisms of Origin.* Adv Exp Med Biol, 2019. **1166**: p. 75-85.
203. O'Donnell, L., *Mechanisms of spermiogenesis and spermiation and how they are disturbed.* Spermatogenesis, 2014. **4**(2): p. e979623.
204. McPherson, S., *Chromatin structure-function alterations during mammalian spermatogenesis : DNA nicking and repair in elongating spermatids.* Eur J Histochem, 1993. **37**: p. 109-128.
205. Marchiani, S., et al., *Spermatozoal Chromatin Structure: Role in Sperm Functions and Fertilization*. 2020. p. 39-55.
206. Henkel, R. and K. Leisegang, *Origins of Sperm DNA Damage*, in *Male Infertility: Contemporary Clinical Approaches, Andrology, ART and Antioxidants*, S.J. Parekattil, S.C. Esteves, and A. Agarwal, Editors. 2020, Springer International Publishing: Cham. p. 361-375.
207. Green, D.R., N. Droin, and M. Pinkoski, *Activation-induced cell death in T cells.* Immunological Reviews, 2003. **193**(1): p. 70-81.
208. Sakkas, D., et al., *Abnormal spermatozoa in the ejaculate: abortive apoptosis and faulty nuclear remodelling during spermatogenesis.* Reproductive BioMedicine Online, 2003. **7**(4): p. 428-432.
209. Łuczaj, W. and E. Skrzydlewska, *DNA damage caused by lipid peroxidation products.* Cell Mol Biol Lett, 2003. **8**(2): p. 391-413.
210. Abbotts, R. and D.M. Wilson, 3rd, *Coordination of DNA single strand break repair.* Free Radic Biol Med, 2017. **107**: p. 228-244.
211. Ranawat, P. and M.P. Bansal, *Apoptosis induced by modulation in selenium status involves p38 MAPK and ROS: implications in spermatogenesis.* Molecular and Cellular Biochemistry, 2009. **330**(1): p. 83-95.
212. Desagher, S. and J.-C. Martinou, *Mitochondria as the central control point of apoptosis.* Trends in Cell Biology, 2000. **10**(9): p. 369-377.
213. Pradeepa, M.M. and M. Rao, *Chromatin remodeling during mammalian spermatogenesis: role of testis specific histone variants and transition proteins.* Society of Reproduction and Fertility supplement, 2007. **63**: p. 1-10.
214. Panner Selvam, M.K. and A. Agarwal, *A systematic review on sperm DNA fragmentation in male factor infertility: Laboratory assessment.* Arab J Urol, 2018. **16**(1): p. 65-76.
215. Gunes, S., M. Al-Sadaan, and A. Agarwal, *Spermatogenesis, DNA damage and DNA repair mechanisms in male infertility.* Reproductive BioMedicine Online, 2015. **31**(3): p. 309-319.
216. Darzynkiewicz, Z., et al., *Conformation of RNA in situ as studied by acridine orange staining and automated cytofluorometry.* Exp Cell Res, 1975. **95**(1): p. 143-53.
217. Love, C., *Sperm Chromatin Structure Assay*, in *Equine Reproductive Procedures*. 2021. p. 629-631.
218. Evenson, D.P., *Sperm chromatin structure assay (SCSA®).* Methods Mol Biol, 2013. **927**: p. 147-64.
219. Fernández, J.L., et al., *Simple determination of human sperm DNA fragmentation with an improved sperm chromatin dispersion test.* Fertil Steril, 2005. **84**(4): p. 833-42.

220. Fernández, J.L., et al., *The Sperm Chromatin Dispersion Test: A Simple Method for the Determination of Sperm DNA Fragmentation.* Journal of Andrology, 2003. **24**(1): p. 59-66.
221. Ankem, M.K., et al., *Novel assay for determining DNA organization in human spermatozoa: implications for male factor infertility.* Urology, 2002. **59**(4): p. 575-8.
222. Agarwal, A. and R. Sharma, *Sperm chromatin assessment*, in *Textbook of assisted reproductive techniques*. 2023, CRC Press. p. 70-93.
223. Pratap, H., et al., *Assessment of Sperm Deoxyribose Nucleic Acid Fragmentation Using Sperm Chromatin Dispersion Assay.* J Pharmacol Pharmacother, 2017. **8**(2): p. 45-49.
224. Ostling, O. and K.J. Johanson, *Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells.* Biochemical and Biophysical Research Communications, 1984. **123**(1): p. 291-298.
225. Singh, N.P., et al., *Abundant alkali-sensitive sites in DNA of human and mouse sperm.* Exp Cell Res, 1989. **184**(2): p. 461-70.
226. Domingo, M., et al., *EXPLORING THE CORRELATIONS BETWEEN SPERM PARAMETERS, TYPES OF SPERM DNA FRAGMENTATION AND PROTAMINE ALTERATIONS.* BIOLOGIA DE LA: p. 25.
227. Ribas-Maynou, J., et al., *Double stranded sperm DNA breaks, measured by Comet assay, are associated with unexplained recurrent miscarriage in couples without a female factor.* PLoS One, 2012. **7**(9): p. e44679.
228. Simon, L. and D.T. Carrell, *Sperm DNA damage measured by comet assay.* Methods Mol Biol, 2013. **927**: p. 137-46.
229. Soler-Ventura, A., et al., *Mammalian Sperm Protamine Extraction and Analysis: A Step-By-Step Detailed Protocol and Brief Review of Protamine Alterations.* Protein Pept Lett, 2018. **25**(5): p. 424-433.
230. Singh, N.P., et al., *A simple technique for quantitation of low levels of DNA damage in individual cells.* Exp Cell Res, 1988. **175**(1): p. 184-91.
231. Ribas-Maynou, J., et al., *Alkaline and neutral Comet assay profiles of sperm DNA damage in clinical groups.* Human Reproduction, 2012. **27**(3): p. 652-658.
232. Muratori, M., et al., *Investigation on the Origin of Sperm DNA Fragmentation: Role of Apoptosis, Immaturity and Oxidative Stress.* Mol Med, 2015. **21**(1): p. 109-22.
233. Agarwal, A., S. Gupta, and R. Sharma, *Measurement of DNA fragmentation in spermatozoa by TUNEL assay using bench top flow cytometer.* Andrological evaluation of male infertility: a laboratory guide, 2016: p. 181-203.
234. Sharma, R., J. Masaki, and A. Agarwal, *Sperm DNA fragmentation analysis using the TUNEL assay.* Methods Mol Biol, 2013. **927**: p. 121-36.
235. Sharma, R.K., et al., *TUNEL as a test for sperm DNA damage in the evaluation of male infertility.* Urology, 2010. **76**(6): p. 1380-6.
236. Zhang, Z., et al., *Predictors of pregnancy outcome for infertile couples attending IVF and ICSI programmes.* Andrologia, 2016. **48**(9): p. 874-881.
237. Xue, L.T., et al., *Effect of sperm DNA fragmentation on clinical outcomes for Chinese couples undergoing in vitro fertilization or intracytoplasmic sperm injection.* J Int Med Res, 2016. **44**(6): p. 1283-1291.
238. Henkel, R., et al., *TUNEL assay and SCSA determine different aspects of sperm DNA damage.* Andrologia, 2010. **42**(5): p. 305-13.
239. Cui, Z.L., et al., *Diagnostic Accuracies of the TUNEL, SCD, and Comet Based Sperm DNA Fragmentation Assays for Male Infertility: a Meta-analysis Study.* Clin Lab, 2015. **61**(5-6): p. 525-35.

240. Chohan, K.R., et al., *Comparison of chromatin assays for DNA fragmentation evaluation in human sperm.* J Androl, 2006. **27**(1): p. 53-9.
241. García-Peiró, A., et al., *Dynamics of sperm DNA fragmentation in patients carrying structurally rearranged chromosomes.* Int J Androl, 2011. **34**(6 Pt 2): p. e546-53.
242. Ribas-Maynou, J., et al., *Comprehensive analysis of sperm DNA fragmentation by five different assays: TUNEL assay, SCSA, SCD test and alkaline and neutral Comet assay.* Andrology, 2013. **1**(5): p. 715-22.
243. Simon, L., et al., *Comparative analysis of three sperm DNA damage assays and sperm nuclear protein content in couples undergoing assisted reproduction treatment.* Human Reproduction, 2014. **29**(5): p. 904-917.
244. LeSaint, C., et al., *Correlation between two sperm DNA fragmentation tests (TUNEL and SCSA) and evaluation of TUNEL assay inter-lab variabiality.* Fertility and Sterility, 2016. **106**(3): p. e297.
245. Alahmar, A.T., R. Singh, and A. Palani, *Sperm DNA Fragmentation in Reproductive Medicine: A Review.* J Hum Reprod Sci, 2022. **15**(3): p. 206-218.
246. Tímermans, A., et al., *DNA fragmentation of human spermatozoa: Simple assessment of single- and double-strand DNA breaks and their respective dynamic behavioral response.* Andrology, 2020. **8**(5): p. 1287-1303.
247. Kobayashi, T., et al., *Protective role of superoxide dismutase in human sperm motifity: superoxide dismutase activity and lipid peroxide in human seminal plasma and spermatozoa.* Human Reproduction, 1991. **6**(7): p. 987-991.
248. Carrell, D.T., et al., *Sperm DNA fragmentation is increased in couples with unexplained recurrent pregnancy loss.* Arch Androl, 2003. **49**(1): p. 49-55.
249. Borini, A., et al., *Sperm DNA fragmentation: paternal effect on early post-implantation embryo development in ART.* Hum Reprod, 2006. **21**(11): p. 2876-81.
250. Puscheck, E.E. and R.S. Jeyendran, *The impact of male factor on recurrent pregnancy loss.* Curr Opin Obstet Gynecol, 2007. **19**(3): p. 222-8.
251. Saxena, P., et al., *Possible role of male factors in recurrent pregnancy loss.* Indian journal of physiology and pharmacology, 2007. **52**: p. 274-82.
252. Gopalkrishnan, K., et al., *Poor quality of sperm as it affects repeated early pregnancy loss.* Arch Androl, 2000. **45**(2): p. 111-7.
253. Chen, C.H., et al., *Apoptosis and kinematics of ejaculated spermatozoa in patients with varicocele.* J Androl, 2004. **25**(3): p. 348-53.
254. Okagbue, H.I., et al., *Trends and usage pattern of SPSS and Minitab Software in Scientific research.* Journal of Physics: Conference Series, 2021. **1734**(1): p. 012017.
255. Pappas, S. and A. Perlman, *Complementary and alternative medicine. The importance of doctor-patient communication.* Med Clin North Am, 2002. **86**(1): p. 1-10.
256. Iserson, K.V., *Principles of biomedical ethics.* Emergency Medicine Clinics, 1999. **17**(2): p. 283-306.
257. Cooper, T.G., et al., *World Health Organization reference values for human semen characteristics.* Hum Reprod Update, 2010. **16**(3): p. 231-45.

Appendices

Appendices-1.1 informed consent form for participation in study

Title: ″Influence of Various Factors on Functional and Molecular Aspects of The Semen Quality Associated with Male Infertility

Study Conducted By: FEMACARE FERTILITY

Investigator: Kale Sudarshan Nagorao

Supervisor: Dr. Ashish Aggarwal, Dr. Jibanananda Mistra

Objective/Purpose of the study: Numerous known and unknown factors affect the semen parameter. our study will help to find out the effects of different factor on semen parameter. our study will help to society to avoid exposer of such factors which will help to increase the semen quality in future.

Who appears to take part in this study?

The couples who are facing the problem in achieving the pregnancy.

How many people are expected to take part in this study?

We are including >1000 subjects.

What will happen to me in this study?

Nothing will happen you. It is a just data base study only your information is included in the study. Confidentiality will be strictly maintained.

How much of my time will be needed to take part in this study?

The study requires to collect Questionnaires from you.it will be over in half hours.

How could I benefit if I take part in this study? How could others benefit?

Not personal profit but your society appears to get the knowledge from our study.

If I want to stop participating in the study, what should I do?

You are free to leave the study at any time. If you leave the study before it is finished, there will be no penalty to you. If you decide to leave the study before it is finished,

please tell one of the persons listed in Section 9. “Contact Information.” If you choose to tell the researchers why you are leaving the study, your reasons may be kept as part of the study record. The researchers will keep the information collected about you for the research unless you ask us to delete it from our records. If the researchers have already used your information in a research analysis it will not be possible to remove your information.

Will my information be used for future research or shared with others?

No, your information is confidential we will keep it with us only.

Who appears to I contact about this study?

Please contact the researchers listed below to:

a. Obtain more information about the study
a. Ask a question about the study procedures
b. Report an illness, injury, or other problem (you may also need to tell your regular doctors)
c. Leave the study before it is finished
d. Express a concern about the study

Principal Investigator: Mr. Sudarshan kale

Email: sudarshan18993@gmail.com

Phone: 9423202323

Procedure: 1) Questionnaires

2)Semen Analysis

3) DFI (optional)

Financial incentives for Participation: You will not be charged for these studies. Also, you will not be paid any form of incentive to participate in this study.

Authorisation to publish results, Privacy and Confidentiality: The results of this study may be published for public interest and scientific purpose. However, your name will not be identified and confidentiality of the data will be maintained.

Consent Statement: I have read the information given above/it has been read to me in vernacular language. All the information regarding this study has been provided to me and I have understood the same. I have been given the opportunity to ask questions and got appropriate answers. I give my consent voluntarily without any force/pressure for participation in this study.

SIGN OF PATIENT.

Appendices-1.2 Questionnaire format

Location of the study site: _____

Date:_____Time of semen collection: _____

Patient identification number: _____

---

1. Age: _____ Weight: _____ Height: _____ BMI: _____

2. What is your ethnic background?

_____Black or Afriappears to Ameriappears to
_____White
_____Hispanic or Latino
_____Ameriappears to Indian or Alaska Native
_____Pacific Islander or Hawaiian
_____Asian
_____Other. Please describe: _____

3. **Medical history:**

a. Do you have any medical problems that you are aware of: ____Yes ____No
b. Have you been treated for any of the disease/STD: ____Yes ____No
   If yes, please specify: _____
c. Have you had any surgery: _____Yes ____No
   If yes, please specify: _____
d. Are you currently taking any medication(s): _____Yes ____No

   If yes, please give the names of the medication(s): _____

1. Have you ever had a urinary tract infection /epididymis/prostatitis/testicular torsion/undescended testis/any serious injury to one or both testis……..?

4. FERTILITY

a. How many partners you have: _____
b. How long you and your partner have been trying to achieve a pregnancy:_____
c. Have you ever achieved a pregnancy with your current partner (Primary or secondary fertility): _____
d. Has your partner been evaluated by any gynecologist: _____Yes ____No

If yes, please specify: _____

5. FAMILY HISTORY:

1) Do you have siblings: _____Yes ____No
If yes, please specify how many brothers and sisters you have: _____
2) Are any of your siblings infertile: _____Yes ____No
3) Are your parents and grandparents are Infertile: _____Yes ____No

6. ENVIRONMENTAL AND OCCUPATIONAL HISTORY:

a) What is your current occupation: _____
4) How long have you been in this occupation? _____
Working hours: _____ Stressful: _____Yes ____No
5) Do you chew tobacco or tobacco products: _____Yes ____No
If yes, tell the frequency_____ and brand name_____
6) Do you smoke any cigarettes (or other tobacco products): _____Yes ____No
If yes, please tell us how many cigarettes (or other products) did you smoke per day:_____, Brand name:_____
b) Are you former smoker for less than 5 yrs or more_____
c) Have you been near someone else who smoked: _____Yes ____No
d) Do you use any drugs currently: _____Yes ____No
If yes, tell the name _____(marijuana/cocaine/heroin/methadone)
e) Do you take alcohol: _____Yes ____No
If yes, frequency_____, Quantity (pack)_____, Brand name_____
f) Have you ever been in contact with pesticide(s): _____Yes ____No
If yes, tell the brand name _____, and duration of exposure_____
g) Have you been exposed to toxic chemicals /heavy metal ions/radiation/industrial solvent on daily basis: _____Yes ____No
If yes, tell the name _____, and duration of exposure_____

7. DIET HISTORY

a. Do you take tea/coffee: _____Yes ____No
If yes, frequency_____/day
b. Do you take cold drinks:_____Yes ____No
If yes, frequency_____/day
c. Do you have water purifier:_____Yes ____No
d. Do you eat fast food frequently:_____Yes ____No

e. Are you a vegetarian or non-vegetarian:_____Yes ____No
If vegetarian, which foods frequently eat_____
If non-vegetarian, Type of nonveg_____, Frequency_____.

f. Do you eat smoked/grilled/Tandoor food:_____Yes ____No

g. Do you take some special food/ supplementary diet in specific season:_____Yes ____No
If yes, please specify_____

8. SEXUAL FUNCTION

a) What is average frequency of intercourse: _____

b) Do you know about her menstrual cycle and fertile period for intercourse:_____Yes ____No

c) Do you require any manual stimulation to maintain erection for successful intercourse:_____Yes ____No

d) Do you have erectile disfunctioning (ED) :_____Yes ____No

9. TESTING

- Semen analysis report
  a. Abstinence period: _____
  b. Volume_____, pH_____, Viscosity_____, Color_____
  c. Count/ml_____, Motility_____, PR_____, NPR_____, IM_____, Morphology_____
  d. Leucocyte_____, Round Cells_____
  e. DFI Test_____

All outcome from test results were maintained in excel sheet with patient code

Appendices-2 Standard operating protocol for semen collection and semen analysis

WHO Guidelines for collection of semen samples

The WHO guidelines describe various procedures for semen sample collection, storage, and processing.

1. Abstinence: The semen sample is collected after maintain the 2-7 days of ejaculation abstinence.
2. Collection: A sterilized, wide-mouthed container is required to collect the semen sample and the container should be non-toxic, non-pyrogenic, and non-spermicidal.
3. Transportation: The sample should be transported to the IVF-lab at 37 °C within 30 minutes of ejaculation.
4. Processing: The sample should be permitted to liquefy for 30 minutes at room temperature and further mixed gently to ensure homogeneity.
5. Examination: The sample should be examined within 60 minutes of collection. The volume, pH, and viscosity should be measured, and the semen should be examined for concentration, motility, and morphology.
6. Storage: If the sample is not examined immediately, it should be stored at 37 °C and examined within 2 hours of collection.
7. Rejection Criteria: Semen samples that contain blood, urine or show signs of bacterial contamination or poor quality should be rejected.

WHO reference values for Semen analysis

A comprehensive semen study is the principal stage in a clinical assessment to measure reproductive capacity in male. The WHO has amended the minor reference limits with confidence intervals (CI) of 95% for the sperm analysis as shown in Table 33 [264].

Table 33: Normal values for semen parameters by WHO including 95% CI

Semen Parameters	**95% CI**	**Standard Values**
Semen concentration	12–16	15 million spermatozoa/ml
Progressive motility	31–34	32%
Volume	1.4–1.7	1.5 ml
Sperm Morphology	3–4	4% normal forms
Total sperm per ejaculation	33–46	39 million spermatozoa

Semen vitality	55–63	58% live
Total sperm motility (progressive and nonprogressive)	38–42	40%

According to WHO guidelines for the examination as well as treating of human semen, published in 2010, the following parameters are considered for semen analysis [257]. As shown in table, the volume of semen should be between 1.5 and 5.0 ml. The pH of semen should be between 7.2 and 8.0. The concentration of sperm in the semen should be at least 15 million sperm per ml. At least 40% of the sperm should be motile (moving). At minimum 4% of the spermatozoa should have a normal shape. At least 58% of the sperm should be alive. The number of white blood cells should be less than or equal to 1 million per ml. The level of fructose in the semen should be above a certain level. Quality of semen is evaluated using these parameters and determine whether a man is fertile. Semen analysis is a primary diagnostic test applied in the assessment of male infertility.

Procedure of Semen analysis

Patient Information

- First check the patient report for viral markers and previous case history (surgery, medications, disease). Check previous semen analysis report if available.

Sample Collection procedure

- The sample must preferably be collected after 2 to 7 days of abstinence, but not longer than 7 days.
- The semen should preferably be collected on site (clinic itself); if this is not possible, the sample must be carried to the IVF laboratory in 45 mins of ejaculation. During this period, the specimen must be kept at body temperature.
- While Collection:
  - Label the container (Name, Abstinence Period, Procedure).
  - Give instructions to the patient for sample collection.
  - Collect the sample by performing masturbation, if this is not possible then appears to perform coitus by using non-spermicidal sterile condoms.

- Wash hands before the procedure.
- Collect the whole sample in the container and avoid spillage as first few drops are important.
- Keep the container on the table and inform reception. Inform if encounters any collection problem or spillage.

Macroscopic Assessment of semen

Before starting assessment label each disposable and consumable with patient name (pipette, slide, etc.)

Take proper precautions before handling the sample such as wear gloves and mask as the sample may contain dangerous infectious agents.

- **Liquefaction**: Keep the sample for liquefaction at 37 ^{0}C for 30-60 mins. (If not liquefied in 60 mins, it should be reported),

  Mucus stands (gelatin) appears to be present in sample which do not have any clinical significance asbut may affect semen examination and therefore should be reported.
- **Appearance**: Report the appearance of the semen within 30 mins of collection: Pearlish White/Opalescent Grey - Normal

  Red – Haematospermia (Presence of blood).

  Yellow - Presence of infection (jaundice) or longer abstinence period.
- **Homogenization**: Mix the sample well by swirling followed by pipetting in and out for 2-3 times.
- **Volume:** Determine the sample volume by using graduated 3 or 5 ml pasteur pipette.
  - 1.4 ml - 6 ml - Normal
  - <1.4 ml - Hypospermia
  - >6 ml - Hyperspermia

  If very little or no semen sample is obtained after sexual climax, you should check for retrograde ejaculation by examining the urine sample obtained immediately after you attain sexual climax.

- **Viscosity**: Allow semen to drop from the pipette tip and observe the length of the thread to check the viscosity of the semen.
    - Normal: semenwill leave the pipette as small discrete drops.
    - Visc + : Thread length of <2 cm
    - Visc ++ : Thread length >2 cm
    - Visc +++ : Clumps and clots present
- **pH** - Check pH by placing a drop of semen on pH paper and match the color with calibrated color code strip.
    - Normal - 7.2 - 8.2
    - Acidic - <7.2
    - Alkaline - >8.2

Preparation for Microscopic Assessment of sperm

- **Make a wet preparation:**
- Label the slide properly with patient name.
- Thoroughly homogenize the sample in the initial container, being careful not to produce air bubbles.
- Place a drop of 10 µl homogenized sample onto a clean microscope slide.
- Carefully place a 22-millimeter x 22-millimeter cover slip directly over the drop, being careful not to trap air bubbles within the cover slip and the slide's surface.
- After the contents stop drifting, wait five seconds to settle the specimen and evaluate the newly prepared wet preparation.
- Assess the slide on 40x by examining multiple high-power fields.
    - **Assessment under the microscope:**
    - At 10x - A brief description of the specimen is given here, revealing any apparent mucus threads, sperm aggregation or agglutination, and whether the spermatozoa are equally distributed throughout the entire process.
    - At 40x - Assess the sperm count, motility, etc.
    - **Count** - Check multiple high-power fields and note the average no of sperms present in one field.
- Normal - >16 million/ml

- Oligozoospermia - <16 million/ml - >5Million/ ml
- Severeoligozoospermia - <5 million/ml
- Cryptozoospermia - Sperms found in pellet after centrifugation
- Azoospermia - No sperms found after centrifugation.

- **Motility** - Count first 100 sperms and divide them into 4 categories depending on their movement and examine this in 10 different fields and note the average.

- Fast progressive (PR) – spermatozoa moving aggressively, either linearly or in a big circle.
- Non-progressive (NPR) – all other forms of active tail movements but lack of progression. Moving at one point.
- Immotile (IM) – no active tail movements.
- Motility Grading (TM) - Progressive (PM) + Non progressive (NP).
- Normal - TM >42% and PM - >30%
- Asthenozoospermia - <42% or PM - <30%.

- **Morphology** - Material required - Stain A (Fixative), Stain B (Eosin), Stain C (Haematoxylin), Glass slide, distilled water, micropipette, semen sample.

- Make a thin smear of sperms on a glass slide.
- Let it to air dehydrated.
- Stain it with Stain A for 8 secs and drain the excess stain (Fixative).
- Stain it with Stain B for 14 secs and drain the excess stain (Eosin).
- Stain it with Stain C for 8 secs and drain the excess stain (Haematoxylin).
- After staining allow it to air dry.
- Then check on 100x and categorize then according to their morphology.
- Normal
- Head defects
- Mid piece defects
- Tail defects
- According to WHO, normal morphology is >=4%
- If the morphology is less than 4%, then it is considered as teratozoospermia.

- **Aggregation** - Aggregation occurs when motile spermatozoa cling to debris, non-sperm cells, or mucus threads.
- **Agglutination** - In particular, motile spermatozoa adhering to one another, either head-to-head, tail-to-tail, or in a combination, is referred to as agglutination.
  - Degree of agglutination:
    - Grade 1 - <10 sperms/agglutinate
    - Grade 2 - >10 to <50 sperms/agglutinate
    - Grade 3 - >50 sperms/agglutinate
    - Grade 4 - all sperms/agglutinate
  - Parts involved:
    - Grade A – (H-H) Head-to-Head
    - Grade B – (T-T) Tail to tail
    - Grade C - Tail tip to tail tip
    - Grade D - Mixed
    - **Viability Test:**
      - Take 10 µl of sample on a glass slide
      - Add 5 µl of eosin nigrosine solution and mix it properly
      - Check it under microscope on 20x before drying
      - The dead sperms will take the stain and be pink coloured whereas the live sperms will remain colorless
      - Score 100 sperms and differentiate them into viable and non-viable
        - Viability, >54% - Normal
        - Viability, <54% - Normozoospermia
      - Pus cells:
        - Take 10ul of semen sample on a glass slide.
        - Add 10ul of WBC reagent solution and mix it properly.
        - Add a coverslip on it and wait for 5 mins.
        - Check it under microscope on 20x.
        - The pus cells will be stained bound in color.
        - Check for no. of brown stained round cells per 100 sperms.
  - WBCs, <1% - Normal

- WBCs, >1% - Pyrospermia

o Round cells: These are immature spermatogonial cells.

o Take 10ul of semen on a glass slide.

o Add a coverslip and wait for 5 secs.

o Check it under microscope on 20x and check for round cells if present.

o Count no of round cells per 100 sperms.

o If round cells are present, report it as present an if not report it as nil.

o **Fructose Test:**

Principle: This test is done when sample is azoospermic. This test is done to check if the patient has obstructive azoospermia or non-obstructive azoospermia.

- Take 200 ul of semen sample in a round bottom tube.
- Add 1:1 ratio (semen: reagent) of seliwanoffs reagent in the tube.
- Heat the mixture for 30 secs and look for the colour change.
- If colour changes to cherry red, then this indicates fructose positive and no change in colour indicates fructose negative.
- If it is fructose positive, then the patient has non-obstructive azoospermia, and if the patient is fructose negative then the patient has obstructive azoospermia.

Appendices-3 SOP For Sperm Chromatin Dispersion method

The SCD test based on the concept that fragmented DNA sperm does not create the typical halo of dispersed DNA loop seen in non-fragmented DNA sperm after acid denaturation as well as nuclear protein elimination. SCD is very easy, fast, and reliable, reproducible technique.

- **Requirement**
  i. Sperm chromatin kit (Solutions (A-B-C-D))
  ii. Sperm Croma Kit of dehydrant (70%, 90%, 100%)

iii. Well liquefied semen sample
iv. Boiling water bath.
v. Distilled water.
vi. Disposables and instruments.

- **Standard Procedure**

a. Label all disposable with patient ID and date.
b. Melt agarose gel in boiling water bath for 3 min.
c. Kept that melted agarose on 37 ^{0}C for 2 min.
d. Add 50 µl of semen sample into agarose gel mix it well and pore this solution on agarose coated glass slide and cover by coverslip.
e. Place this slide into refrigerator for 5 min
f. Remove the coverslip carefully without disturbing the layer of agarose.
g. Apply solution A for 7 min at room temperature.
h. Drain excess of solution A and apply solution B for 22 min at 22 ^{0}C.
i. Drain out excess of solution B and rinse the slide by distilled water.
j. Apply 70%, 90% and 100% respectively at interval of 2 min for each step.
k. Wipe out back of slide and allow air-drying.
l. Then mix solution C (150 µl) and solution D (250 µl) and apply on slide for 3-5 min.
m. Drain out excess of stain solution and rinse the slide by distilled water.
n. Observe under 40x and then 100x objectives.
o. Evaluate 400 spermatozoa with big halo or without halo

Outcomes

p. DFI is measured with the following formula
q. $SDFI\ (\%) = \frac{\text{No.Of sperms having fragmented DNA}}{\text{Total no of counted sperm}} x\ 100$

Appendices- 4 Abbreviations

ART	Assisted reproductive technology
SA	Semen analysis
TM	Total motility
PR	Progressive motility
NPR	Non progressive motility
IM	Immotile
Morpho	Morphology
DFI	DNA fragmentation index
SCD	Sperm chromatin dispersion
CASA	Computer assisted semen analysis
ANOVA	Analysis of variation
CI	Confidence interval
ED	Erectile dysfunction
HSF	Heat shock protein
IVF	Invitro fertilization
ICSI	Intracytoplasmic sperm injection
PH	Percentage of hydrogen
BMI	Body mass index
SAP	Sexual abstinence period
SF	Sexual frequency
hiPSCs	Human induced pluripotent stem cells
HPG	hypothalamus-pituitary-gonadal
AAS	anabolic-androgenic steroids
ECS	endogenous appears tonabinoid system
STD	Sexually transmitted disease
ASIH	anabolic steroid-induced hypogonadism
EPC	eppin protein complex
HFD	high-fat diet
CETN1	centrin 1
CSPP1	centrosome and spindle pole-associated protein 1
TUBB4B	tubulin beta-4B chain

List of Publication

1) Sudarshan Kale, Sarthak Gandhi, Ashish Aggarwal, Jibanananda Mishra, “The Impact of Medical History, Dietary Habits, and Type of Drinking Water on Semen Quality”, **Communicated**
2) Sudarshan Nagorao kale, Jibanananda Mishra, Ashish Aggarwal “Impact of Sexual Abstinence Period on Functional and Molecular Semen Parameters” **communicated.**
3) Sudarshan Kale, Sarthak Gandhi, Ashish Aggarwal, Jibanananda Mishra, “Factors Affecting Semen Quality a Comparative Study on Functional and Molecular Aspects Across Age BMI And Lifestyle”, International journal of infertility and fatal medicine, **Accepted** on: 16 November 2024
4) Sudarshan Kale, Sarthak Gandhi, Sakshi Godghase , Ashish Aggarwal “Seasonal effects on semen parameters affecting male infertility”, International journal of infertility and fatal medicine. **Accepted** on: 01 October 2024
5) Sudarshan Nagorao kale, Jibanananda Mishra, Ashish Aggarwal “Exploring Risk Factors and Functional Aspects of Semen Quality in Male Infertility: The Intersection of Reproductive Health and Lifestyle”, Afriappears to Journal of Biological Sciences, May 2024, Volume 6, Issue 10.
6) Sudarshan Nagorao kale, Jibanananda Mishra. “An Appraisal of Behavioural and Non-Behavioural Factors Influencing the Male Infertility: Narrative Approach”, International Journal of Applied Biology and Pharmaceutical Technology. June 2023, Volume 14, Issue 1.
7) Sudarshan Kale, Jibanananda Mishra, “Influence and possible consequences of exogenous factors on male fertility”, Journal of Emerging Technologies and Innovative Research, January 2019, Volume 6, Issue 1.

List of Conference

Conference with presentation

1) Sudarshan Kale, Jibanananda Mishra, Ashish Aggarwal, “Impact of sexual abstinence on functional and molecular semen parameter”, International conference on recent advances in fundamental and applied sciences (RAFAS), April 2024, LPU, Phagwara, Punjab
2) Sudarshan Kale, Jibanananda Mishra, Ashish Aggarwal, **“**Influence of various factors on molecular aspects of the semen quality associated with male infertility”, International congress of academy of clinical embryologist (ACE), September 2023, Bengaluru, Karnataka
3) Sudarshan Kale, Jibanananda Mishra,” Influence of semen viscosity on outcome of IUI” Indian society of assisted reproduction (ISAR), November 2018, Jaipur, Rajasthan

Conference attended

4) Sudarshan Kale “academy of clinical embryologist ACE e-Summit conference, May 2021, Online
5) Sudarshan Kale “Embryo-Tech 1.0”, June 2022, Online
6) Sudarshan Kale ACE conference, “International congress of academy of clinical embryologist” , September 2024, Pune, Maharashtra

List of Workshops

1) Sudarshan kale, “Insight into re-implantation genetic testing- invasive and non-invasive methods” at “International congress of academy of clinical embryologist”, September 2024, Pune, Maharashtra.