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The Effects of Tobacco and Cannabis Consuming on Male Fertility: A Comprehensive Analysis of Sperm Quality, Chromatin integrity, Genetic Variations, and Oxidative Stress

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The Effects of Tobacco and Cannabis Consuming on Male Fertility: A Comprehensive Analysis of Sperm Quality, Chromatin integrity, Genetic Variations, and Oxidative Stress

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MT-CO1: mitochondrially encoded cytochrome c oxidase I

MT-CO2: mitochondrially encoded cytochrome c oxidase II

MT-CO3: mitochondrially encoded cytochrome c oxidase III

CASA: computer assisted sperm analysis

CI: confidence intervals.

THC: Tetrahydrocannabinol

CBD: Cannabidiol

DNA: deoxyribonucleic acid

SNPs: Single Nucleotide Polymorphisms

FSH: follicle stimulating hormones.

LH: luteinizing hormone.

GnRH: Gonadotrophin-Releasing Hormone

IARC: International Agency for Research on Cancer

COPD: chronic obstructive pulmonary disease

WHO: World Health Organization

PAH: polycyclic aromatic hydrocarbons

ECS: endocannabinoid system

ATP: adenosine triphosphate

ADP: adenosine diphosphate

GTP: guanosine triphosphate

CB: Cannabinoid-Rezeptor

RR: risk ratios

CS: Cannabis Smoker

TS: Tobacco Smoker

NS: Non-Smoker

NADH: Nicotinamide adenine dinucleotide

FADH: flavin adenine dinucleotide

MT-DNA: Mitochondrial DNA

OS: oxidative stress

ROS: reactive oxygen species

ETC: electron transport chain

OXPHOS: oxidative phosphorylation

8-OHG: 8-hydroxyguanosine

HIV: human immunodeficiency virus

IVF: In Vitro fertilization

ELISA: Enzyme-Linked Immunosorbent Assay

NCBI: National Center for Biotechnology Information

ABSTRACT

Recent comprehensive meta-analysis research has uncovered a notable reduction in sperm concentration and total sperm counts among men from North America, Europe, and Australia between 1973 and 2011. This decline, estimated at 50–60%, affects men regardless of their fertility status, with no signs of stabilization in recent years. These findings strongly suggest a significant deterioration in male reproductive health, with implications that extend beyond fertility concerns (Levine H et al., 2017). Given the magnitude of this global shift, the most compelling explanation is one that universally affects all populations involved. There is no doubt about the significant impact that new lifestyle changes can have on us.

It is well-documented that tobacco smoking adversely affects a broad spectrum of physiological functions. Additionally, the use of tobacco and cannabis presents an escalating threat to male reproductive health. Although the prevalence of tobacco smoking declined over time among men in reproductive age, the use of other inhaled products, such as cannabis have increased. Therefore, understanding their potential deleterious effects on male fertility is crucial for the well-being of future generations.

Numerous studies over recent decades have indicated a correlation between smoking tobacco or cannabis and a decline in sperm quality, potentially leading to male infertility. Tobacco smoking, a widespread global occurrence, considerably impacts not only an individual's well-being but also their genetic makeup. Over the past few years, comprehensive research has explored the intricate relationship between tobacco exposure and the expression of numerous genes. Recently cannabis consuming has shifted following the legislation of marijuana in various countries and states, an imperative public health concern has emerged that affect not only to users but also to their offspring.

The perception of Cannabis toxicity and safety in society, politics, and even scientific circles has changed dramatically in recent decade. Consequently, a few questions have been raised regarding the effects of marijuana (Cannabis sativa) on human physiology and reproductive health in the short and long term.

Therefore, the purpose of this study was to determine the effects of tobacco and cannabis use on sperm quality, chromatin integrity, and oxidative damage among men Previously engaged in annual use of tobacco or cannabis. And to investigate, nonetheless, the damage that tobacco and cannabis and their constituents on sperm mitochondrial genetic variants in the Cytochrome C Oxidase 1, 2 and 3 genes (MT-CO1, MT-CO2, and MT-CO3). Specifically on the alterations in the cytochrome oxidase encoded gene present within sperm mitochondria.

Initially, 10 semen samples have undergone an in Vitro study by analyzing semen parameters (volume, sperm count, motility), within 1 hour after collection, according to WHO criteria 2020. For accurate results, Computer Assisted Sperm Analysis (CASA) system was used to determine sperm parameters. Each sample was exposed to 1 mL; 10 µg/mL of THC, THC-COOH, and CBD dissolved in methanol. All samples were also exposed to 1 mL pure methanol as a control group to eliminate the potential effects of methanol on sperm cells. Several smears (10 µL) of each sample were taken in order to assess the functionality of sperm's chromatin integrity by employing a Chromomycin (CMA_3) and DNA fragmentation (Acridine Orange) assays.

Then, a total of 116 samples were collected from men aged less than 40 years at Prince Rashid Bin Al Hassan Hospital (PRBH) in Irbid, Jordan. The samples were divided into three groups: Group 1 - Non-smokers (control group, n=38), Group 2 (n=39), - Tobacco heavy smokers, more than 10 cigarettes a day for at least 5 years, and Group 3 - Cannabis smokers (n=37) with at least 4 joints in a week for more than 3 years.

Chromatin integrity and DNA damage was assessed by using Chromomycine CMA_3 and Acridine Orange staining. The 8-OHG RNA assay was employed to measure oxidative damage. Besides, the whole genome of spermatozoa was extracted using a QIAamp DNA Mini Kit and the mitochondrial DNA was amplified by using the REPLI-g Mitochondrial DNA Kit. Polymerase chain reaction (PCR) was used to amplify the MT-CO1, MT-CO2 and MT-CO3 gene. Then, samples were purified and sequenced using the Sanger method.

The treatment of sperm cells with 10µL of THC, THC-COOH, and CBD resulted in a significant decline in total sperm motility upon exposure to THC and CBD ($P \leq 0.001$ and $P \leq 0.003$, respectively). Progressive motility was notably reduced ($P \leq 0.001$), and the average number of immotile sperm significantly increased ($P \leq 0.003$) following incubation with THC and CBD, respectively. However, only a slight reduction in total sperm motility was observed after incubation with THC-COOH.

In vivo, in the other hand, sperm count, and progressive motility showed no significant difference between the investigated groups (non-smokers, tobacco smokers, and cannabis smokers). However, the mean value of immotile spermatozoa and morphologically abnormal spermatozoa increased among smokers and cannabis smokers compared to non-smokers (58,92 % for Tobacco smokers, 68,66 % for cannabis smokers and 51,73 % for non-smokers). (5.02 for tobacco smokers, 2.26 for cannabis smokers and 7.46 for non-smokers). In addition, the

mean value of chromatin condensation of spermatozoa showed a statistically significant difference among non-smokers (15 % ± 15.4), tobacco smokers (25.3 % ± 14.9; P ≤ .001), and cannabis smokers (37,13 % ± 20.1; P ≤ .001).

A cumulative sum of 22 single nucleotide polymorphisms (SNPs) was detected in the mitochondrial cytochrome c oxidase subunit 1 (Mt-Co1) gene, with a distribution of 37,8 %, 64,1 %, and 54 % among non-smoker, tobacco smoker, and cannabis smoker groups, respectively.

On the other hand, 17 Single Nucleotide Polymorphisms (SNPs) have been identified within the mitochondrial-encoded Cytochrome C Oxidase II (MT-CO2) gene, with a proportion of 21,62 %, 28,2 %, and 43,24 % among non-smoker, tobacco smoker, and cannabis smoker groups, respectively.

In the mitochondrial-encoded cytochrome c oxidase III gene (Mt-Co3), a total of 20 single nucleotide polymorphisms (SNPs) were identified, with a distribution of 54,05 %, 64,1 %, and 51,35 % among the investigated groups - non-smokers, tobacco smokers, and cannabis smokers, respectively. Nevertheless, none of these SNPs were significantly different between the three groups.

This study demonstrates that tobacco and cannabis smoking have a negative effect on sperm parameters, chromatin condensation (Sperm maturity) and DNA integrity of spermatozoa and should be avoided in men of reproductive age. It highlights the importance as well of promoting education and awareness regarding cannabis use and its extensive implications, affecting not only individual health but also the well-being of future generations.

ZUSAMMENFASSUNG

Jüngste umfassende Meta-Analysen haben eine bemerkenswerte Reduktion der Spermienkonzentration und der Gesamtspermienzahl bei Männern aus Nordamerika, Europa und Australien zwischen 1973 und 2011 aufgedeckt. Dieser Rückgang, der auf 50–60% geschätzt wird, betrifft Männer unabhängig von ihrem Fruchtbarkeitsstatus, ohne Anzeichen einer Stabilisierung in den letzten Jahren. Diese Ergebnisse deuten stark auf eine bedeutende Verschlechterung der männlichen reproduktiven Gesundheit hin, mit Auswirkungen, die über Fruchtbarkeitsbedenken hinausgehen (Levine H et al., 2017). Angesichts des Ausmaßes dieser globalen Verschiebung ist die überzeugendste Erklärung eine, die alle beteiligten Bevölkerungen universell betrifft. Es besteht kein Zweifel an den erheblichen Auswirkungen, die neue Lebensstiländerungen auf uns haben können. Es ist gut dokumentiert, dass das Rauchen von Tabak eine breite Palette physiologischer Funktionen negativ beeinflusst. Darüber hinaus stellt der Gebrauch von Tabak und Cannabis eine zunehmende Bedrohung für die männliche reproduktive Gesundheit dar. Obwohl die Prävalenz des Tabakrauchens im Laufe der Zeit bei Männern im fortpflanzungsfähigen Alter gesunken ist, hat der Gebrauch anderer eingeatmeter Produkte wie Cannabis zugenommen. Daher ist es entscheidend für das Wohlergehen zukünftiger Generationen, ihre potenziell schädlichen Auswirkungen auf die männliche Fruchtbarkeit zu verstehen. Zahlreiche Studien der letzten Jahrzehnte haben einen Zusammenhang zwischen dem Rauchen von Tabak oder Cannabis und einem Rückgang der Spermienqualität aufgezeigt, der potenziell zur männlichen Unfruchtbarkeit führt. Tabakrauchen, eine weltweit verbreitete Erscheinung, beeinträchtigt nicht nur das Wohlbefinden eines Einzelnen, sondern auch seine genetische Beschaffenheit erheblich. In den letzten Jahren haben umfangreiche Forschungen die komplexe Beziehung zwischen Tabakexposition und der Expression zahlreicher Gene untersucht. In jüngster Zeit hat sich der Cannabiskonsum nach der Legalisierung von Marihuana in verschiedenen Ländern und Bundesstaaten verändert, und es ist ein unerlässliches öffentliches Gesundheitsproblem aufgetreten, das sich nicht nur auf die Benutzer, sondern auch auf ihre Nachkommen auswirkt. Die Wahrnehmung der Cannabistoxizität und -sicherheit in Gesellschaft, Politik und sogar in wissenschaftlichen Kreisen hat sich in den letzten Jahrzehnten dramatisch verändert. Daraus ergeben sich einige Fragen zu den Auswirkungen von Marihuana (Cannabis sativa) auf die menschliche Physiologie und reproduktive Gesundheit auf kurze und lange Sicht. Das Ziel dieser Studie war es daher, die Auswirkungen von Tabak- und Cannabiskonsum auf die Spermienqualität, die Chromatinintegrität und den oxidativen Schaden bei Männern, die zuvor

jährlich Tabak oder Cannabis konsumiert haben, zielt die Studie darauf ab, die Schäden zu untersuchen, die Tabak und Cannabis sowie ihre Bestandteile auf mitochondriale genetische Varianten der Spermien in den Cytochrom-C-Oxidase-1-, 2- und 3-Genen (MT-CO1, MT-CO2 und MT-CO3) verursachen. Speziell auf die Veränderungen im durch das Zytochromoxidase - kodierte Gen innerhalb der Spermienmitochondrien. Zunächst wurden 10 Samenproben einer in-vitro-Studie unterzogen, indem die Spermaparameter (Volumen, Spermienzahl, Beweglichkeit) innerhalb von 1 Stunde nach der Entnahme analysiert wurden, gemäß den WHO-Kriterien 2020. Zur Erzielung genauer Ergebnisse wurde ein computergestütztes Spermaanalyse (CASA) -System zur Bestimmung der Spermaparameter verwendet. Jede Probe wurde mit 1 mL; 10 µg/mL THC, THC-COOH und CBD in Methanol gelöst, belichtet. Alle Proben wurden auch mit 1 mL reinem Methanol als Kontrollgruppe belichtet, um die potenziellen Auswirkungen von Methanol auf Samenzellen zu eliminieren. Mehrere Abstriche (10 µL) jeder Probe wurden genommen, um die Funktionalität das Chromatin verdichten geschlechtsreifer Spermatozoen unter Verwendung von Chromomycin blaue (CMA3) und Desoxyribonukleinsäure Spaltung (Acridinorang) -Analysen zu bewerten. Anschließend wurden insgesamt 116 Proben von Männern unter 40 Jahren in Prince Rashid Bin Al Hassan Hospital (PRBH) in Irbid, Jordanien, gesammelt. Die Proben wurden in drei Gruppen unterteilt: Gruppe 1 - Nichtraucher (Kontrollgruppe, n = 38), Gruppe 2 (n = 39) - Tabak starke Raucher, mehr als 10 Zigaretten pro Tag für mindestens 5 Jahre, und Gruppe 3 - Cannabis Raucher (n = 37) mit mindestens 4 Joints pro Woche für mehr als 3 Jahre. Die Chromatin verdichten und der DNA schaden wurde durch Verwendung von Chromomycin CMA_3 und Acridin Orang-Färbung bewertet. Der 8-OHG-RNA-Test wurde verwendet, um den oxidativen Schaden zu messen. Darüber hinaus wurde das gesamte Genom der Spermatozoen unter Verwendung eines QIAamp-DNA-Mini-Kits extrahiert und die mitochondriale DNA unter Verwendung des REPLI-g mitochondrial DNA Kit amplifiziert. Die Polymerase-Kettenreaktion (PCR) wurde verwendet, um das MT-CO1-, MT-CO2- und MT-CO3-Gen zu amplifizieren. Anschließend wurden die Proben gereinigt und unter Verwendung der Sanger-Methode sequenziert. Die Behandlung von Samenzellen mit 10 µL THC, THC-COOH und CBD führte zu einem signifikanten Rückgang der Gesamtspermienmotilität nach der Exposition gegenüber THC und CBD ($P \leq 0{,}001$ bzw. $P \leq 0{,}003$). Die progressive Motilität wurde signifikant reduziert ($P \leq 0{,}001$) und die durchschnittliche Anzahl von unbeweglichen Spermien stieg signifikant an ($P \leq 0{,}003$) Anschließend zeigte sich nur eine leichte Reduktion der Gesamtspermienmotilität nach der Inkubation mit THC-COOH. In vivo hingegen zeigten die Samenanzahl und die progressive

Motilität keinen signifikanten Unterschied zwischen den untersuchten Gruppen (Nichtraucher, Tabakraucher und Cannabisraucher). Der Mittelwert der unbeweglichen Spermatozoen und morphologisch abnormen Spermatozoen erhöhte sich jedoch bei Rauchern und Cannabisrauchern gegenüber Nichtrauchern (52,42% für Tabakraucher, 65,13% für Cannabisraucher und 44,59% für Nichtraucher). Bei den Rauchern für Tabak und Cannabisraucher betrug er entsprechend (5,02 bzw. 2,23 und 7,34). Außerdem zeigte der Mittelwert der Chromatinkondensation der Samenzoen unter den Nichtrauchern (14,68 ± 15,64), den Tabakrauchern (25,28 ± 14,86; $P \leq 0,024$) und den Cannabisrauchern (34,83±18,39; $P \leq 0,001$) einen statistisch signifikanten Unterschied.

Eine kumulative Summe von 22 einzelnen Nukleotidpolymorphismen (SNPs) wurde im mitochondriellen Zytochrom-c-Oxidase-Untereinheit 1 (Mt-Co1) -Gen gefunden, mit einer Verteilung von 37,8 %, 64,1 %, and 54 % bei Nichtrauchern, Tabakrauchern und Cannabisrauchern, respektive.

Auf der anderen Seite wurden 17 Einzel-Nukleotid-Polymorphismen (SNPs) im mitochondriell codierten Zytochrom-C-Oxidase-II (MT-CO2) -Gen identifiziert, mit einem Anteil von 21,62 %, 28,2 %, and 43,24 % bei Nichtrauchern, Tabakrauchern und Cannabisrauchern, respektive.

Im mitochondriell codierten Zytochrom-c-Oxidase-III-Gen (Mt-Co3) wurden insgesamt 20 einzelne Nukleotid-Polymorphismen (SNPs) identifiziert, mit einer Verteilung von 54,05 %, 64,1 %, and 51,35 % bei den untersuchten Gruppen - Nichtrauchern, Tabakrauchern und Cannabisrauchern, respektive. Dennoch gab es keine signifikanten Unterschiede zwischen den drei Gruppen in Bezug auf diese SNPs.

Diese Studie zeigt, dass Tabak- und Cannabisrauchen einen negativen Einfluss auf Spermienparameter, Chromatin-Kondensation (Spermienreife) und die DNA-Integrität von Spermatozoen haben und bei Männern im reproduktiven Alter vermieden werden sollten. Sie unterstreicht auch die Bedeutung der Förderung von Bildung und Bewusstsein in Bezug auf den Cannabiskonsum und seine umfangreichen Auswirkungen, die sich nicht nur auf die individuelle Gesundheit, sondern auch auf das Wohlergehen zukünftiger Generationen.

1. INTRODUCTION

1.1 Tobacco

Approximately 14% of the global reproductive population is affected by infertility (Fishel et al., 2000). Various lifestyle choices and pathological conditions can influence male fertility. Notably, 50% of subfertility cases are attributed to idiopathic causes. These may result from genetic factors, including gene defects, karyotype abnormalities (Thielemans, B et al., 1998), and single nucleotide polymorphisms (SNPs) (Krausz et al., 2015). Additionally, environmental and lifestyle factors such as, dietary habits, alcohol intake and tobacco or cannabis use, play a significant role in reproductive health (Fishel et al., 2000). Tobacco is widely recognized for its adverse health effects. The history of tobacco and its health implications have been well documented, with studies linking its use to a range of serious illnesses. Notably, researchers Musk and De Klerk have highlighted the irony of tobacco's history, where it was once used for purported health benefits, only for its consumption to become one of the major causes of preventable diseases (Musk et al., 2003). Tobacco smoking is particularly detrimental to the development of lung cancer, chronic obstructive pulmonary disease (COPD), heart disease, and stroke, as detailed by the World Health Organization in its comprehensive status report on the global impact of tobacco (WHO 2019). Additionally, according to R. West's findings, even smokeless forms of tobacco carry significant health risks, underscoring the importance of addressing all types of tobacco use (West Robert 2017). The Population Assessment of Tobacco and Health (PATH) has further reinforced the need for stringent regulation and public health interventions to reduce tobacco consumption and protect human health (Hyland A et al., 2017). Tobacco smoking is currently responsible for approximately 30% of all cancer deaths in developed countries (Vineis et al., 2004). In addition, smoking causes a greater number of deaths from cardiovascular, chronic obstructive pulmonary and degenerative diseases. In 2000, 4.8 million premature deaths worldwide were attributed to smoking, of which 2.4 million in developing and 2.43 million in developed industrialized countries (Ezzati et al., 2004), numbers expected to increase to 10 million a year by 2030 (Ezzati et., 2004), nonetheless, cigarette smoking appeared to contribute significantly towards impairment of sperm motility. Tobacco smoke is an aerosol containing about 1000 particles/mL, consisting of highly porous carbonaceous polymeric material with adsorbed heavy metals, polycyclic aromatic hydrocarbons (PAH), N-nitrosamines and various

other organic chemicals. The phase of tobacco smoke contains at least 3,500 chemical compounds and a high proportion of them are toxic, carcinogens or mutagens, (e.g. benzene, 2-napthylamine, 210Po, 226Ra, 228Ra, nickel, cadmium, benzopyrene, etc) (Hecht, 1999). There are at least 55 carcinogens in cigarette smoke that have been evaluated by the International Agency for Research on Cancer (IARC) with “sufficient evidence for carcinogenicity” (Hoffman and Hoffmann 1997; Hoffmann and Wynder 1986).

1.2 Cannabis

The historical utilization of medicinal cannabis can be traced back to ancient civilizations. Physicians across diverse regions, such as Asia and the Middle East, incorporated cannabis into various medications for pain relief and other therapeutic purposes (Friedman et al., 2017). In the 19th century, Western medicine began to recognize the therapeutic potential of cannabis, which saw a peak in medical use during the last decade of that century (Zuardi A., 2006).

Since then, there has been significant progress in terms of drug administration techniques, with the development of various cannabinoid-based pharmaceuticals. However, despite these advances, smoking cannabis remains a prevalent consumption method due to the immediate effects experienced by users (Goodman et al., 2019).

In the United States, cannabis was initially grown for its hemp -fibers-, but its recreational use in the early 20th century led to the Marijuana Tax Act of 1937, which imposed heavy taxes on its sale, effectively limiting its use and possession to medical and industrial purposes (Musto et al., 1972).

The Controlled Substances Act of 1970 classified cannabis as a Schedule I drug, indicating a high potential for abuse and no accepted medical use. However, this classification has been challenged due to emerging research highlighting the plant's medicinal properties (Gettman J, 2001).

Today, there is a global trend towards the legalization and decriminalization of cannabis, especially for medicinal use. Various states in the U.S. and countries around the world are changing their laws, influenced by new research, public opinion shifts, and the potential

economic benefits from cannabis taxation (Shover et al., 2019). Despite these changes, cannabis remains illegal in many parts of the world. Below are some intriguing statistical insights regarding cannabis:

►Approximately 147 million individuals, or 2.5% of the global population, consume cannabis annually. This contrasts with just 0.2% who use cocaine and another 0.2% who use opiates. During the current decade, cannabis abuse has escalated more rapidly than that of cocaine and opiates (WHO 2016).

►Nearly 76% of people believe that marijuana is less harmful than tobacco (Popova et al., 2017), and 67% think it is safer than prescription painkillers (Corroon et al., 2017).

►The Centre for Disease Control and Prevention reported that nearly 40% of high school students have tried marijuana. (Eaton et al., 2010).

►19% of teenage drivers admit to driving while under the influence of marijuana (Asbridge et al., 2005).

► In Germany and according to the results of the 2021 alcohol survey on alcohol, smoking and cannabis by the Federal Centre for Health Education, the proportion of young people aged 12 to 17 who consumed cannabis in the last twelve months before the survey was 4.6 percent in 2011 increased to 7.6 percent in 2021 (Orth, B. & Merkel, C 2022).

►According to the 2021 Epidemiological Addiction Survey, 8.8 percent of all adults aged 18 to 64 used cannabis at least once in the last 12 months; that's around 4.5 million people. The results of this study show that one in four cannabis users have problematic use (Rauschert & Christian, et al 2022).

Cannabis, also known as marijuana, is derived from the dried leaves and flowers of the Cannabis sativa plant. People consume it for its psychoactive effects, such as relaxation and mild euphoria, or for its physiological benefits. Upon consumption, cannabis releases cannabinoids that interact with the body's endocannabinoid system (ECS), binding to cannabinoid receptors. In the United States and many European countries, marijuana is the most used illicit drug and is gaining popularity as both a recreational substance and a medicinal aid, particularly among men of reproductive age. However, our understanding of the long-term effects of exposure to marijuana is very poor and contradictory results have been reported. Also, cannabis smoking has garnered significant attention in recent years, particularly for its potential impact on various aspects of human health. Cannabis sativa and

Cannabis indicia are the most widespread and best-characterized species of cannabis; extracts of both plants contain phytocannabinoids of therapeutic interest, such as Δ9 - tetrahydrocannabinol (THC) and cannabidiol (CBD) (El Sohly et al., 2014; WHO., 2018). Cannabis varies substantially in the level of its two major cannabinoids: delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD). Levels of CBD can range from almost zero to up to 40%. THC is often associated with deleterious effects such as impaired cognition, anxiety, and psychotic-like symptoms, whereas CBD has been associated with antipsychotic and anxiolytic-like effects, and might even offer neuroprotection (Morrison et al., 2009; McPartland et al., 2015).

It is crucial to mention that cannabis smoke has been shown to have numerous toxicants and carcinogens including acrylonitrile (a potential carcinogen carbon monoxide cardiovascular toxin), 6 and 1,3, -butadiene (a carcinogen) (Wei et al., 2016). Very few published studies have examined cannabis smoke metabolites in humans (Wei et al., 2018).

At the same time, cannabidiol (CBD)-rich products are undergoing significant growth within the North American market. That (unlike THC) does not produce impairment when used alone. Compared with dried herbs, CBD is traditionally present in higher concentrations in orally ingested oils and capsules. A broader use of CBD products as natural health products also has emerged in topical creams, often at subclinical concentrations. Anecdotal evidence further suggests that there is an increasing demand for dried herbs that has higher levels of CBD or products with balanced levels of CBD and THC. This is likely due to preliminary research suggesting that CBD may have anti-psychotic effects and/or moderate some of the impairment produced by THC (Hoffman and Hoffmann 1997; Hoffmann and Wynder., 1986).

For centuries, the active components found in Cannabis sativa have been utilized for both recreational and medicinal reasons. The remarkable benefits of cannabis-based medicines are widely recognized, and their clinical use is now expanding across the globe. With its rich history and increasing popularity, it's clear that cannabis has a significant role to play in modern medicine (Bonini et al., 2018). Following the legislation of marijuana in various countries and states, an imperative public health concern has emerged that affect not only users but also their offspring.

Also, cannabis is most widely misused substance, as used recreational drug, and has been subject to significant reclassification and medical application. In 2004, it was downgraded from Class B to a Class C drug. Its potential use in treating multiple sclerosis, alongside

synthetic THC (Dronabinol) which is used in the United States and several European countries to alleviate AIDS-related cachexia, reduce nausea from cancer chemotherapy, and address chronic pain and anxiety, has fueled widespread misconceptions about its legality and health impacts (Acevedo B et al., 2007).

In 2021, 44% of adults surveyed believed smoking marijuana every day is safer than smoking tobacco every day, compared to about 37% in 2017 (Chambers et al., 2023).

The past few years have witnessed a dramatic change regarding Cannabis toxicity and safeness on the eyes of society, politics and even scientific field. Therefore, several critical questions about the short and long-term impact of marijuana (Cannabis sativa) on human body and psychic abilities have risen. Presently, Comprehensive discussions on global political and societal dimensions are taking place at both the state and federal levels regarding the potential legalization of Cannabis sativa or the employment of its derivatives for the alleviation of intractable medical conditions (Szutorisz et al., 2016).

Although, Cannabis (marijuana) is the most widely used illicit drug globally, and According to a survey conducted by the National Survey on Drug Use and Health (NSDUH), in 2019, approximately 19.4 million adults in the United States used marijuana in one month only. And according to a study published in the journal Addiction estimated that in 2015, global Cannabis consumption was between 128 and 238 million people (Freeman et al., 2015).

Consequently, in recent decades, there has been a growing concern about the impact of tobacco and cannabis smoking on male reproductive health. Understanding their potential effects on fertility is crucial for the well-being of future generations.

1.3 Sperm cell

The word "sperm" is derived from the Greek word sperma (meaning "seed"). The seed that contains life inside of it and with a convenient time and environment grows into living being.

Sperm, the male reproductive cell, is haploid, containing 23 chromosomes. These chromosomes can combine with the 23 chromosomes from the female egg to create a diploid cell. In mammals, the sperm matures within the testicles, is stored in the epididymis, and later released via the penis.

The primary function of sperm is to reach and merge with the egg, delivering two key sub-cellular structures: (i) the male pronucleus, which carries the genetic material, and (ii) the centrioles, which are essential for organizing the cell's microtubule cytoskeleton.

The mammalian sperm cell can be divided into three parts:

Head: contains the nucleus with densely coiled chromatin, surrounded anteriorly by a thin, flattened sac called the acrosome, which contains enzymes used for penetrating the female egg.

Tail: also called the flagellum, is the longest part and capable of wave-like motion (thanks to its actin and myosin protein enriched structure) that propels sperm for swimming and aids in penetration the egg process.

The midpiece: has a central filamentous core with many mitochondria spiralled around it, used for ATP production through the journey into female cervix, uterus, and uterine tubes (Boitrelle et al., 2013).

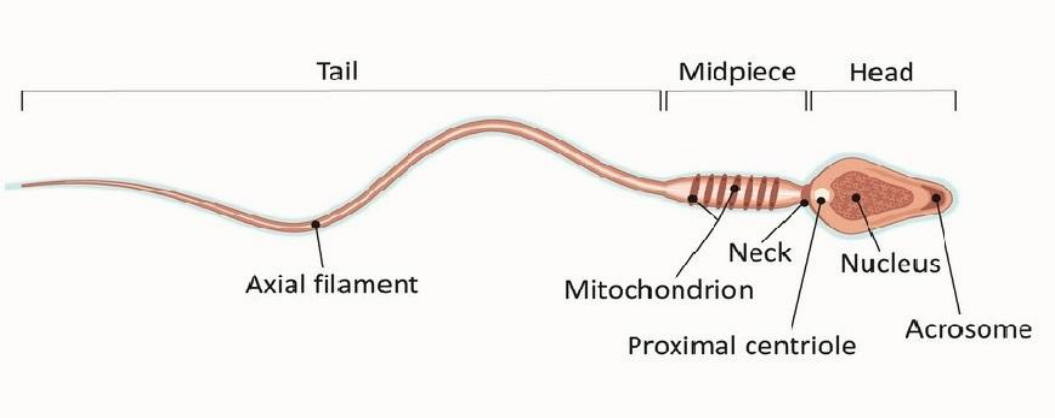


Figure 1; Sperm structure and anatomy shows the Three parts [head, midpiece and tail] (Asadi 2020)

Spermatozoon has almost no cytoplasm, its DNA therefore very sensitive and can be easily damaged. It is still unclear how exactly chromatin damage occurs in human spermatozoa, but there is consensus on various of events that could influence the integrity of sperm chromatin at the time of spermatogenesis, such as Environmental stress, gene mutations, Leukocytospermia

(increasing number of leukocytes that present in seminal plasma) and external factors (Lehti et al., 2017).

1.4 Spermatogenesis and Spermiogenesis

Spermatogenesis is the process by which sperm are formed. Starting at puberty, Leydig cells in the testes begin producing androgens under the influence of two hormones: Follicle-Stimulating Hormone (FSH) and Luteinizing Hormone (LH). The release of FSH and LH is, in turn, stimulated by Gonadotrophin-Releasing Hormone (GnRH), which is produced by the hypothalamus (Szmelskyj & Aquilina, 2014).

On the other hand, spermatogenesis represents an intricate and highly specialized procedure reliant upon the supportive microenvironment facilitated by testicular Sertoli and Leydig cells. The initial cells in this pathway are called spermatogonia, these immature cells are all derived from stem cells in the outer wall of the seminiferous tubules. The stem cells are composed almost entirely of nuclear material (diploid “2n”), they begin their process by multiplying in order to duplicate themselves in process known as mitosis. Half of the new cells remain as stem cells so that there is a constant source of additional germ cells, and the other half goes on to develop into primary spermatocytes, herein where the first meiosis takes place to produce a secondary spermatocyte, thus undergoing meiosis II. Provided that, two spermatids (haploid “1n”) are generated from each secondary spermatocyte, resulting in a total of four spermatids. Lastly these spermatids developed into mature spermatozoa by a process called Spermiogenesis, which is the final stage of spermatogenesis. After the process reaches its final stage, the mature sperm cells will be transferred to the epididymis where they are stored until ejaculation.

The entire process has been reported as taking approximately 74 days (Szmelskyj & Aquilina, 2014). The proliferation and differentiation of the male germ cells and the intra-testicular and extra-testicular mechanisms of the spermatogenesis regulation can be disturbed at each level (Jarow et al., 2002).

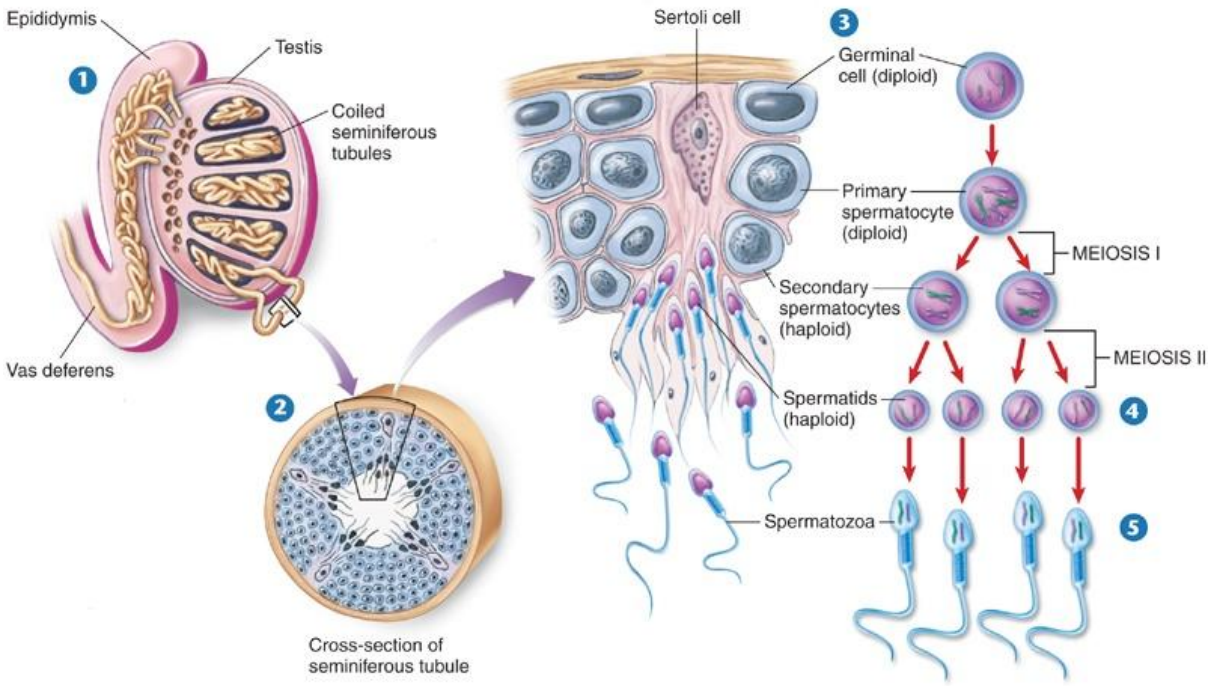


Figure 2; Spermatogenesis occurs within the testes, specifically inside the seminiferous tubules. Here, germ cells mature into primary spermatocytes, which are diploid. These cells then undergo meiosis to transform into haploid spermatids. Following this, the spermatids evolve into motile spermatozoa, also known as sperm. This complex process is supported by Sertoli cells—non-germinal cells embedded in the seminiferous tubule walls—which play a crucial role in converting spermatids to spermatozoa and facilitating the overall progression of spermatogenesis. (spermatogenesis 01-2018).

On the other hand, spermatogenesis represents an intricate and highly specialized procedure reliant upon the supportive microenvironment facilitated by testicular Sertoli and Leydig cells. The detection of cannabinoid receptors on these cellular components points toward a potential function for cannabinoids in maintaining the equilibrium of molecular signaling within this microenvironment. For instance, CB2 receptors are located on Sertoli cells, and modulating this receptor has demonstrated involvement in inducing apoptosis in these cells. Moreover, Leydig cells exhibit the presence of cannabinoid receptor CB1, which instigates a localized decrease in testosterone production, potentially influencing spermatogenesis (Duca et al., 2019).

1.5 Human sperm chromatin

The formation of sperm cell nuclear DNA is a unique process, which differs from the body cells nuclear DNA construction by the replacement of somatic cell-type histones with protamines (sperm-specific basic nuclear proteins), which leads to more condensed packaged chromatin; at least six times more condensed than body cells chromosomes.

This highly condensed packaging keeps the chromatin compact, stable, thereby protecting the genetic integrity and facilitating the transport of the paternal genome through the male and female reproductive tracts.

Spermatozoon has almost no cytoplasm. Its DNA, therefore, is highly sensitive and can be damaged easily. It is still unclear how exactly chromatin damage occurs in human spermatozoa, but there is a consensus about various events that could influence the integrity of sperm chromatin at the time of spermatogenesis, such as environmental stress, gene mutations and leukocytospermia (increasing the number of leukocytes in the seminal plasma) (Fuentes-Mascorro et al., 2000). There have been different methods developed to detect abnormalities or DNA damage in sperm chromatin.

1.6 Mitochondria

Mitochondria are known as the cell's powerhouses. These organelles are the principal source of adenosine triphosphate (ATP) – an energy-packed molecule that fuels vital cellular processes – in eukaryotic organisms that don't use photosynthesis (Rizzuto et al., 2012).

Structurally, a mitochondrion is encased by a double membrane, creating four separate compartments, each compartment plays a unique role: the outer membrane isolates the mitochondrion from the surrounding cytosol, the porous outer mitochondrial membrane allows ions and small uncharged molecules to pass freely through its protein pores, eliminating any potential difference across it. The intermembrane space. is similar to the cytosol with respect to ions and small molecules. The inner membrane contrasts sharply as a stringent barrier to diffusion. Here, only specialized transport proteins can carry specific ions and molecules across—this selectivity results in an electrochemical potential of approximately 180 mV. Consequently, the inner mitochondrial membrane is the functional barrier to the passage of small molecules between the cytosol and the matrix and maintains the proton gradient that

drives oxidative phosphorylation. These potential forms the basis for oxidative phosphorylation on this inner boundary. A process harnessed to produce ATP in a series of complex protein-driven reactions, the inner membrane features numerous folds called cristae that envelop the matrix area (Mannella, 2008). While the matrix serves as the crucial hub for mitochondrial DNA replication, transcription, protein biosynthesis, and a host of enzymatic processes (Kukat et al., 2011, McCarron et al., 2013). Mitochondria operate semi-autonomously within cells. (Kühlbrandt, 2015).

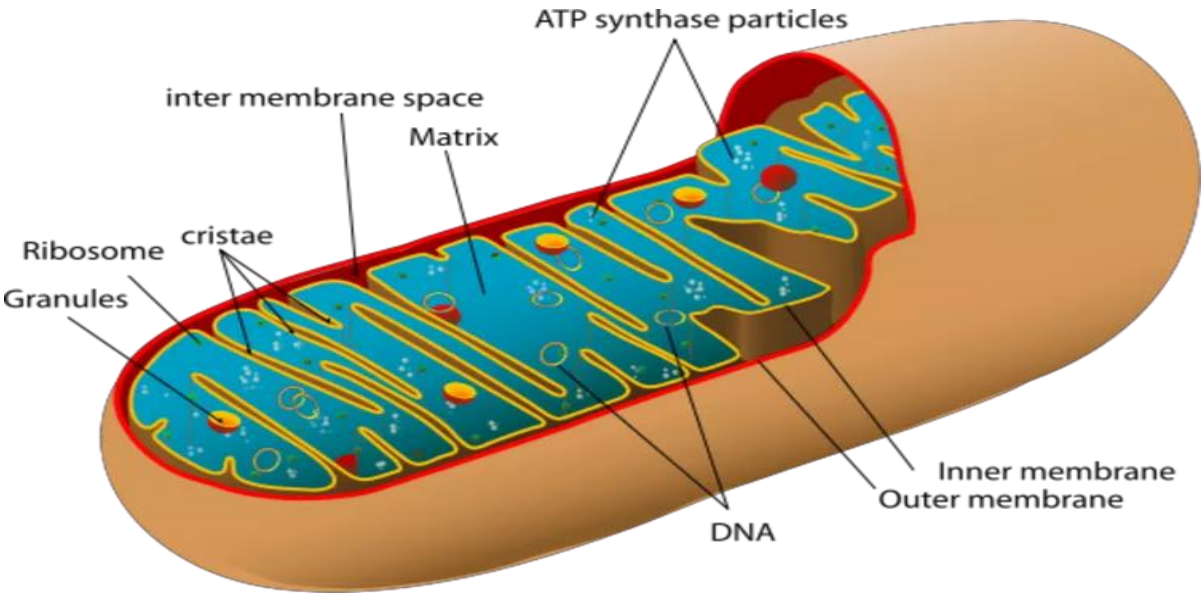


Figure 3; Diagrammatic structural features of a mitochondrion. [Adapted from (Bailey & Regina 2023)]

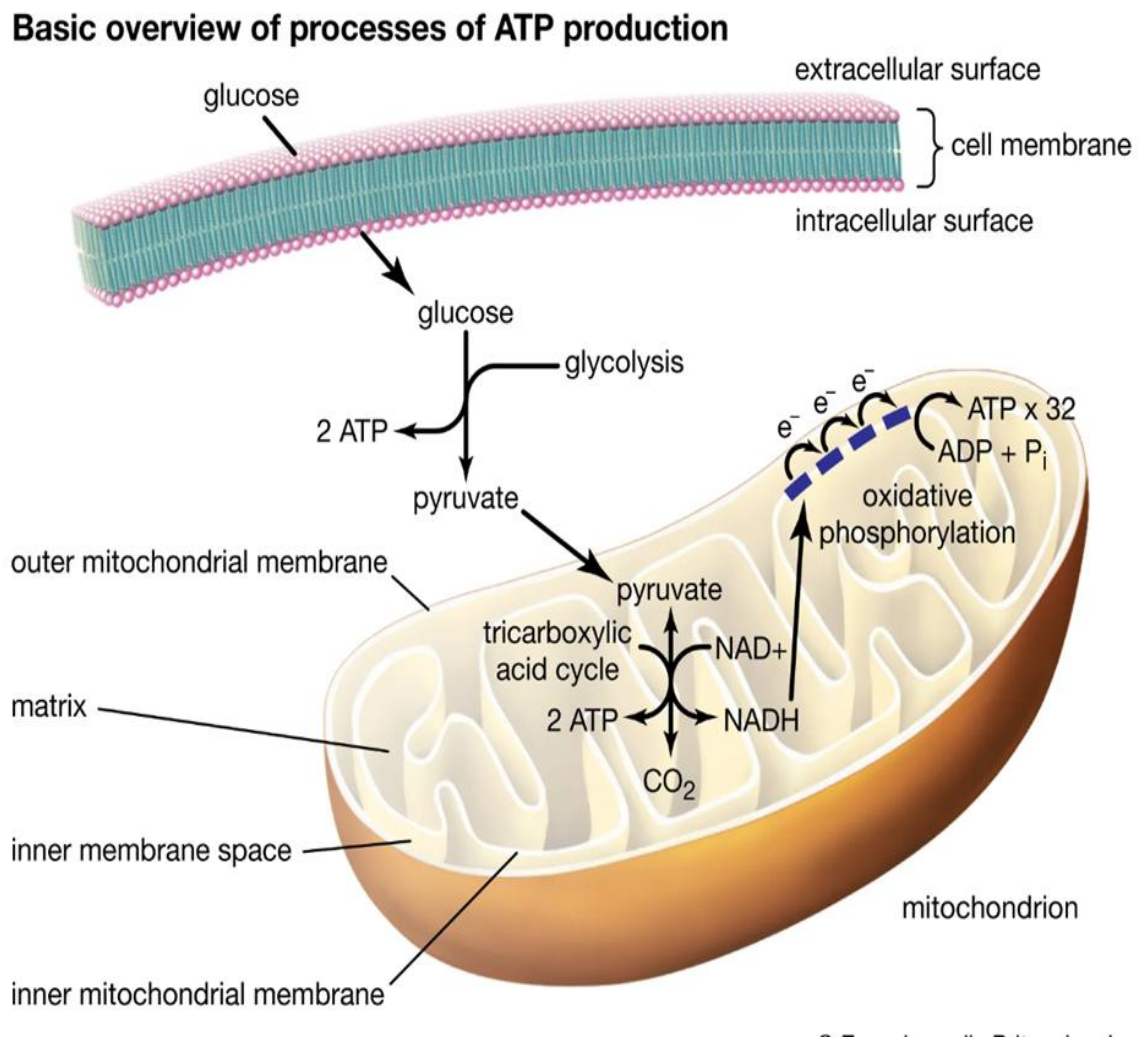


Figure 4; Basic Overview of processes of ATP production

1.6.1 Cellular respiration:

In the cytosol, glycolysis initiates the breakdown of glucose into two molecules of pyruvate. Concurrently, nicotinamide adenine dinucleotide (NAD+) is reduced to NADH, generating ATP in the process. The pyruvate molecules then travel into the mitochondrion's matrix, where the enzyme pyruvate dehydrogenase facilitates their conversion to acetyl-CoA (Maechler, 2006). Following this, acetyl-CoA enters the citric acid cycle (also known as the Krebs cycle), where it combines with citrate and undergoes a series of oxidation reactions.

As citrates are metabolized, electrons are transferred to electron carriers NADH and flavin adenine dinucleotide (FADH2). The energy released during these reactions is harnessed by these carriers and transferred to the electron transport chain (Alabduladhem & Bordoni, 2021).

The final phase of cellular respiration is oxidative phosphorylation, which completes the energy extraction from glucose.

1.6.2 Oxidative phosphorylation:

Oxidative phosphorylation is the primary mechanism by which mitochondria generate ATP. This process encompasses the ATP synthase complex (complex V) along with four other oxidoreductase complexes—NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), cytochrome c reductase (complex III), and cytochrome c oxidase (complex IV). These critical complexes reside in the inner mitochondrial membrane. Complexes I and II are responsible for transferring electrons from NADH or FADH2 to ubiquinone, whereas complex III transmits electrons from ubiquinol to cytochrome c, which is situated in the intermembrane space. Finally, complex IV facilitates the transfer of electrons from cytochrome c to molecular oxygen (Chaban et al., 2014).

Adenosine triphosphate (ATP), a molecule packed with energy, powers fundamental cellular operations, including force generation necessary for muscle contraction and cell division; tasks like protein biosynthesis, folding, and degradation; and establishing and preserving membrane potentials. Remarkably, an average adult human body synthesizes an immense amount of ATP—approximately 50 kilograms daily. Mitochondrial ATP synthase catalyzes the conversion of adenosine diphosphate (ADP) and phosphate ions into ATP (Osellame et al., 2012). In regions of the cell where energy consumption occurs, ADP and Pi are products of ATP breakdown.

Beyond their role in cellular respiration and ATP production, mitochondria undertake numerous vital functions which include generating NADH and guanosine triphosphate (GTP) via the citric acid cycle; biosynthesizing amino acids, heme groups, and iron-sulfur clusters; as well as creating phospholipids for building cellular membranes. They also participate in calcium signaling, manage oxidative stress responses, and serve as overall hubs for cellular signaling (Friedman & Nunnari 2014).

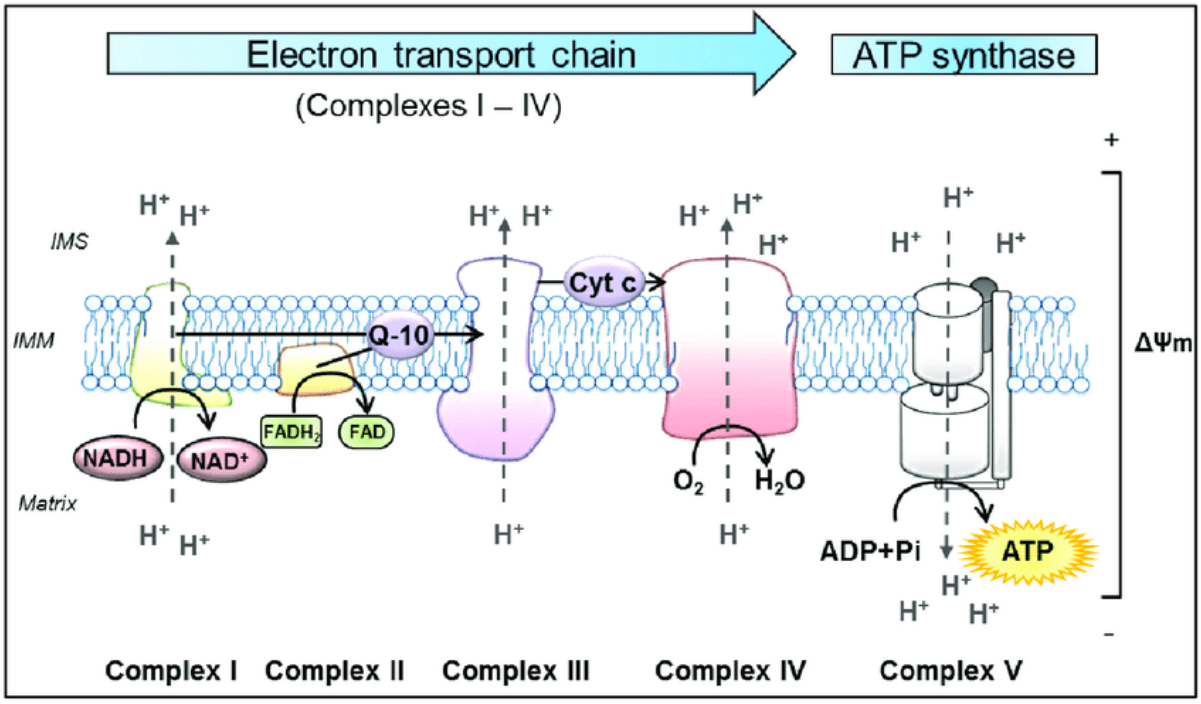


Figure 5; Simplified diagram of oxidative phosphorylation (Szabo et al., 2020)

1.6.3 Mitochondria and oxidative stress:

Oxidative stress (OS) arises when there's an imbalance between reactive oxygen species (ROS) and antioxidants, leading to various cellular disruptions. DNA mutations within the mitochondria, interference with the respiratory chain, and changes in mitochondrial membrane permeability are possible consequences. Additionally, excessive ROS can affect calcium homeostasis and degrade proteins and lipids, thereby weakening the mitochondria's defenses (Guo et al., 2013). Complexes I and III of mitochondrial respiration are considered primary sources of superoxide and other ROS (Hollensworth et al., 2000). Macromolecules such as proteins, lipids, and DNA are vulnerable to hydroxyl radical-induced damage.

Hence, mitochondrial DNA damage is linked to compromised functions of complexes I and/or III. Superoxide formation is tied to the heightened reduction of O2 electrons (Van Houten et al., 2006), and the resulting oxidative damage to mitochondrial DNA heightens risks of genomic instability, metabolic disturbances, and cellular harm (Guo et al., 2013).

Damage to mitochondrial DNA may also reduce certain electron transport chain proteins' expression, which promotes a cycle of escalating ROS production and organelle deterioration that ultimately leads to cell death through apoptosis (Van Houten et al., 2006).

The electron transport chain is notably susceptible to Nitroxyl (NO^-) and peroxynitrite ($ONOO^-$) damage, which affects several metabolic enzymes like cytochrome c oxidase, NADH dehydrogenase, and ATP synthase through protein oxidation and nitration (Andreazza et al., 2010).

ROS exposure can deactivate iron-sulfur (Fe-S) centers in complexes I, II, and III, impairing mitochondrial energy output (Ghezzi & Zeviani, 2012).

ROS not only targets mitochondrial components but also damages spermatozoa's nuclear DNA. Elevated ROS levels can harm sperm functionality by impairing motility and impeding fertilization processes (Kumar et al., 2009).

1.6.4 Mitochondrial DNA (mt-DNA):

Encased within the mitochondrial matrix, mitochondrial DNA (mt-DNA) is maternally inherited and exhibits a conserved structure and genetic configuration across mammals, as noted by (Chiaratti et al., 2020; Farge et al., 2019). This compact and circular double-stranded DNA consists of about 16.6 kilobases divided into heavy (H) and light (L) strands. The heavy strand encodes two rRNA molecules, 14 tRNAs, and 12 polypeptides, while the light strand encodes eight tRNAs and a single polypeptide. These 13 proteins are essential to the oxidative phosphorylation enzyme complexes (Schon et al., 2012).

It's important to note that mt-DNA replication is rapid but lacks a robust repair mechanism. Consequently, as (Bahrehmand Namaghi & Vaziri 2017) found in their study, the mutation rate of mt-DNA significantly surpasses that of nuclear DNA—by 10 to 100 times. Additionally, (Ashok et al., 2014) demonstrated that sperm cells have inadequate internal antioxidant mechanisms, making them more susceptible to oxidative damage.

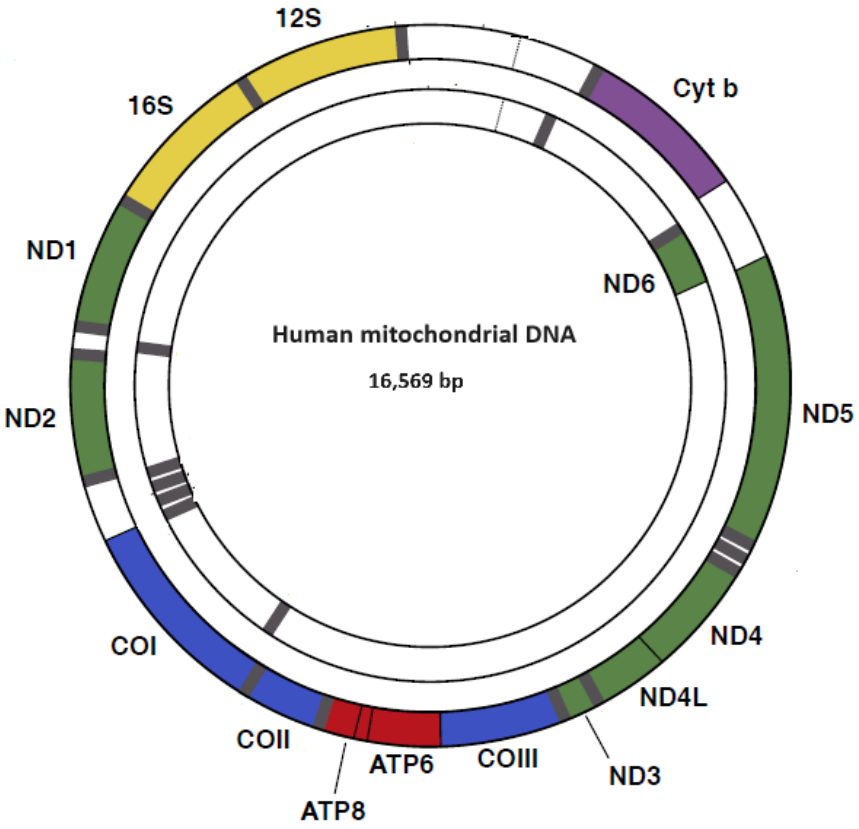


Figure 6; Illustrates the human mitochondrial DNA genomic structure comprising various genes and regulatory regions. Adapted from (Picard et al., 2016).

The 13 protein subunits encoded by mt-DNA are part of complexes I, III, IV, and V of the electron transport chain (Chinnery & Patrick F 2006):

Complex I (NADH dehydrogenase) have seven mt-DNA-encoded subunits.

Complex III (cytochrome b complex) has one mt-DNA-encoded subunit.

Complex IV (cytochrome c oxidase) has three mt-DNA-encoded subunits (I, II, and III).

Complex V (ATP synthase) has two mt-DNA-encoded subunits (6 and 8)

Table 1; Respiratory chain complexes I - V, as encoded by the mitochondrial genome

Complex	No. subunits	No. subunits encoded on mitochondrial genome
I NADPH dehydrogenase	>41	7
II Succinate dehydrogenase	4	0
III Cytochrome bc1	11	1
IV Cytochrome c oxidase	13	3
V ATP synthase	14	2

1.6.4.1 Complex I (NADH dehydrogenase): Is the first and largest enzyme complex in the electron transport chain (ETC) of mitochondrial oxidative phosphorylation. It is located in the inner mitochondrial membrane and plays a crucial role in cellular respiration; the process cells use to make energy in the form of ATP. The primary function of Complex I is to catalyze the oxidation of NADH, a carrier of electrons and protons generated during the Krebs cycle, and the reduction of ubiquinone (also known as coenzyme Q10), which is a lipid-soluble electron transporter within the mitochondrial membrane (Jiménez-Gómez, B et al., 2023).

Malfunctions in Complex I can lead to a range of mitochondrial diseases because of their critical role in energy production. Deficiencies in Complex I activity can result in conditions such as Leber's hereditary optic neuropathy (LHON) and mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS). Moreover, Complex I is a major site of reactive oxygen species (ROS) production, which can lead to oxidative stress and has been implicated in various age-related diseases and neurodegenerative disorders. On the other hand, understanding the structure and function of Complex I can lead to the development of drugs aimed at controlling the effects of mitochondrial diseases (Jiménez-Gómez, B et al., 2023).

1.6.4.2 Complex III (cytochrome b complex): is the third complex in the series of electron transporters within the mitochondrial electron transport chain (ETC). It plays a pivotal role in aerobic cellular respiration and is located in the inner mitochondrial membrane (Gao et al., 2003).

The primary function of Complex III is to catalyze the electron transfer from ubiquinol (reduced coenzyme Q10) to cytochrome c, a small heme protein that is soluble in the intermembrane space. This process is crucial for the continuation of the electron transport chain (Crofts & Antony 2004).

Mutations in the genes encoding the subunits of Complex III can lead to various mitochondrial disorders, which can have a wide range of symptoms due to the critical role of this complex in

energy production. Diseases associated with Complex III deficiencies include exercise intolerance, muscle weakness, and certain multisystem disorders (Gil Borlado et al., 2010).

Antimycin A is a known inhibitor of Complex III, and it's often used in scientific research to study ETC. Understanding the intricacies of Complex III function and its inhibitors has implications for drug development, particularly for conditions that involve mitochondrial dysfunction (Skulachev & Vladimir 1996).

1.6.4.3 Complex IV (cytochrome c oxidase): is the fourth and final enzyme in the mitochondrial electron transport chain (ETC). It plays a vital role in the process of oxidative phosphorylation. Complex IV is located in the inner mitochondrial membrane and is responsible for catalyzing the transfer of electrons from cytochrome c to molecular oxygen (O2), which is the final electron acceptor in the ETC (Castresana, J., et al 1994).

Cytochrome c oxidase is a large transmembrane protein complex that consists of multiple subunits. In mammals, it typically has 14 subunits, with three of them (I, II, and III) being encoded by mitochondrial DNA and the remaining ones by nuclear DNA (Balsa et al., 2012; Zong, S et al, 2018). The complex also contains several metal prosthetic sites, including two heme groups (a and a3) and two copper centers (Cu_A and Cu_B). These metal centers play a crucial role in the enzyme's catalytic action (Tsukihara., et al 1995).

The main function of Complex IV is to receive electrons from cytochrome c and use these electrons to reduce molecular oxygen to water. Here's how the process works:

Electron Transfer: Cytochrome c, which has been reduced by Complex III, binds to Complex IV and transfers its electrons to the Cu_A center of Complex IV.

Reduction of Oxygen: The electrons are passed through the heme a to the heme a3 and Cu_B center, where molecular oxygen binds. The oxygen is then fully reduced to water.

Proton Pumping: At the same time, Complex IV pumps' protons from the mitochondrial matrix to the intermembrane space, contributing to the proton motive force that is used to produce ATP (Ludwig et al., 2001).

Defects in the genes encoding Complex IV subunits or assembly factors can result in cytochrome c oxidase deficiency, which is one of the most common causes of mitochondrial disease in humans. This can lead to a wide spectrum of clinical manifestations, such as muscle weakness, lactic acidosis, and neurodegenerative conditions (Zee et al., 2006).

Complex IV is also a target for certain toxic compounds, such as cyanide, carbon monoxide, and azide, which can bind to the heme a3-Cu_B center and block oxygen reduction, leading to cellular asphyxiation (Nicholls et al., 2013). Understanding the function and structure of Complex IV is thus not only important for fundamental biological research but also for medical and pharmacological applications.

1.6.4.4 Complex V (ATP synthase): is the enzyme responsible for the synthesis of adenosine triphosphate (ATP), the primary energy carrier in cells. It is located in the inner mitochondrial membrane of eukaryotic cells and the plasma membrane of prokaryotic cells (Junge et al., 2015). ATP synthase is the final enzyme of the oxidative phosphorylation pathway. The main function of ATP synthase is to convert the energy of the proton motive force into ATP (Haraux t al., 2019).

Dysfunction in ATP synthase can lead to a range of mitochondrial diseases, as ATP production is essential for cell survival and function. For example, genetic mutations affecting ATP synthase subunits can cause neuromuscular diseases and other conditions related to energy metabolism (Junge et al., 2015).

1.6.5 Mitochondrial Spermatozoa:

The spermatozoon contains approximately 50-75 mitochondria in its midpiece. These mitochondria share a similar structure and function with those found in somatic cells. They generate energy for sperm movement and possess their own DNA, known as mitochondrial DNA (mt-DNA) (Ankel-Simons & Cummins 1996).

In a typical somatic cell, there are approximately 5-10 copies of mitochondrial DNA (mt-DNA) per mitochondrion and between 1,000 and 5,000 copies in the cell itself (Giles et al., 1980). In a spermatozoon, there are roughly 50-75 mitochondria, with each harboring an average of one mt-DNA copy. Conversely, an oocyte houses around 100,000 to 400,000 mitochondria, each containing an average of one mt-DNA copy. Under physiological conditions, a cell's mt-DNA molecules exhibit a uniform state known as 'homoplasmy.' The preservation of homoplasmy is crucial for maintaining normal mitochondrial function through the coordinated expression of mitochondrial and nuclear genes. However, under specific circumstances, a cell may display two or more types of mt-DNA molecules - a state called 'heteroplasmy' (Jansen & de Boer 1998; Steuerwald et al., 2000)

While cells gain efficient energy through oxidative phosphorylation (OXPHOS). However, this process also generates reactive oxygen species (ROS), which can be detrimental to cellular health. The proximity of mitochondrial DNA (mt-DNA) to the respiratory chain complexes makes them susceptible to attack by ROS. Unlike nuclear DNA, mt-DNA lacks histone protection against physical and chemical damage and possesses relatively limited DNA repair mechanisms within the mitochondria. Consequently, the mt-DNA mutation rate is significantly elevated (40-100-fold in certain genes) compared to nuclear DNA (Pesole et al., 1999).

In human spermatozoa, an average of one mitochondrion contains a single copy of mitochondrial DNA (mt-DNA). This mt-DNA sequence is congruent with that of the somatic cells; however, the DNA repair activity in sperm is lower, or even non-existent, compared to somatic cells. Consequently, despite the shorter lifespan and mitotic origin (from spermatogonia) of mature sperm, mt-DNA mutations tend to accumulate rapidly. This observation underscores the essential role of maternal inheritance of mt-DNA and the elimination of sperm mt-DNA in the fertilized egg. Inversely, since sperm mt-DNA is not transmitted to offspring, there is no requirement for sperm mitochondria to repair or eliminate aberrant mt-DNA. As a result, it has been reported that sperm mitochondria lack an inherent mt-DNA repair mechanism (Reynier et al., 1998; Cummins., 1998).

Sperm motility represents a critical factor for spermatozoa progression towards the site of fertilization and subsequent successful fertilization. The mitochondrial genome plays a pivotal role in mature sperm morphogenesis and flagellar motility following ejaculation (Rajender et al., 2010; Nakada et al., 2006). Furthermore, mitochondria, commonly acknowledged as the cellular powerhouse, possess their distinct genome encoding 13 proteins (Shamsi et al., 2008). The human mitochondrial genome exhibits a noteworthy compact and circular structure, consisting of 16,569 base pairs. Mutation rates within mitochondrial DNA (mt-DNA) are relatively high, attributed to the absence of histones and DNA repair mechanisms (Alexeyev et al., 2013). Consequently, mutations that arise within the mitochondrial genome contribute significantly to particul ,ar human genetic disorders. It has been documented that mt-DNA mutations correlate with specific forms of male infertility, such as POLG locus mutations in mt-DNA polymerase (Rovio et al., 2001). Additionally, a high prevalence of single-nucleotide polymorphisms (SNPs) within mt-DNA has been observed in semen samples possessing poor sperm quality (Holyoake et al., 2001; Dahadhah et al., 2021). Numerous studies have demonstrated that mt-DNA mutations within sperm may result in diminished sperm motility and eventual male infertility (Y. L et al., 2020; Al Smadi et al., 2021).

Alterations in the mitochondrial genome can compromise several sperm functions, including motility. Some human mt-DNA variants have been proposed to be responsible for a decreased mitochondrial OXPHOS and, consequently, for reduced spermatozoa motility. However, there are contrasting results due to specific technical and interpretational controversies. Nonetheless, there is a consensus that alterations in the mitochondrial genome can compromise several sperm functions, including motility (Durairajanayagam et al., 2021). These studies and more elevates our recognition of how epigenetic modifications can be influenced by environmental factors such as smoking, cannabis and other drug consumption.

1.7 The effect of tobacco smoking on Spermatozoa

Tobacco smoke is comprised of numerous toxic and mutagenic compounds, among which nicotine, a potent psychoactive substance, is present. Notably, nicotine and its primary metabolite cotinine can traverse the blood-testis barrier, subsequently inflicting varying degrees of damage upon germ cells (Omolaoye et al., 2022). Tobacco use remains prevalent worldwide. According to the World Health Organization, 22.3% of the global population, 36.7% of men and 7.8% of women, used tobacco in 2020 (WHO., 2021).

Cigarette smoking is a recognized health hazard, and the highest prevalence of smokers is in young men of reproductive age (Sobinoff et al., 2014). It was estimated that 5.5% of the global population aged 15–64 have used cannabis recreationally (World Drug Report 2019). According to the World drug Report 2019: 35 million people worldwide suffer from drug use disorders while only 1 in 7 people receive treatment. The correlation between tobacco smoke and both cardiovascular- and cancer-related morbidity has been well-established; additionally, recent research has underscored the detrimental effects of tobacco smoke on male fertility (Pizzol et al 2021; Sharma et al., 2016).

Tobacco is comprised of numerous toxic and mutagenic compounds, among which nicotine, potent psychoactive substance, and its primary metabolite cotinine can traverse the blood-testis barrier, subsequently inflicting varying degrees of damage upon germ cells (Omolaoye et al., 2022). Furthermore, existing studies have substantiated the notion that tobacco smoke can act as both a mutagen and an an-eugen within germ cells/spermatozoa (Marchetti et al., 2011 and Linschooten et al., 2013).

There is a pile of evidence suggest that tobacco smoking has a negative impact on male fertility. While there may be some debate surrounding its effects on sperm motility, it is widely recognized that it can lead to reductions in sperm concentration, morphologically normal spermatozoa, and altered protein expression in addition to genetic and epigenetic anomalies within spermatozoa (Pereira et al., 2014). Furthermore, existing studies have substantiated the notion that tobacco smoke can act as both a mutagen and an aneugen within germ cells/spermatozoa (Marchetti et al., 2011; Linschooten et al., 2013).

Also, Smoking is closely linked to abnormalities in histone-to-protamine transition and with alteration of protamine expression in human spermatozoa. Patients who smoke possess a higher proportion of spermatozoa with an alteration of the histone to protamine ratio than patients who do not smoke (Hamad et al., 2014).

Tobacco smoking affects significantly not only an individual's health but also his or her genetic profile. In recent years, extensive research has delved into the profound interplay between tobacco exposure and the expression of numerous genes. For example, a study conducted on mice by Rangasamy and his research team, it was discovered that tobacco exposure led to the expression of over 1,000 genes in the pulmonary region. Subsequent subacute cigarette smoke exposure resulted in a decrease in the differential expression of 40% of pulmonary genes, while sub chronic and chronic cigarette smoke exposure further affected over 80% of the genes within the lung tissue (Rangasamy et al., 2009).

In another very interesting research study conducted by Huang et al., they have elucidated the connection between tobacco smoking and the consequential telomere shortening and DNA damage observed in embryonic stem cells, and how it may culminate in increased genomic instability and neoplasia susceptibility. In light of these findings, telomere biology emerges as a promising avenue for further exploration concerning tobacco-induced genomic alterations (Huang et al., 2013). In a further illustration of the manner in which tobacco consumption may modify an individual's genomic profile, a study conducted by Alkhaled and his colleagues at Saarland University examined the human sperm methylation profile of cigarette smokers in comparison to a control group. The research identified 11 CpG sites exhibiting a significant disparity in DNA methylation between the two groups (Alkhaled et al., 2018).

Various studies have provided insights into the multiple pathways through which tobacco smoking affects sperm cells. One salient mechanism is the direct toxicological impact of the constituents of cigarette smoke on testicular function (Mostafa et al., 2006). For instance, it has

been discovered that nicotine impairs spermatogenesis by inducing oxidative stress, DNA damage, and apoptosis within germ cells, leading to reduced sperm count and motility (La Vignera et al., 2016 and Jorsaraei et al., 2008).

These outcomes can be attributed to the augmented generation of reactive oxygen species (ROS), which subsequently provokes oxidative stress (OS), DNA impairment, and germ cell apoptosis. Additionally, tobacco smoking is known to increase seminal reactive oxygen species (ROS) levels, which disrupt sperm functionality and results in decreased fertilization capacity (Pasqualotto et al., 2008).

While ROS is essential for certain physiological processes (Haque et al., 2014), excessive accumulation can lead to DNA strand fractures, unsaturated lipid peroxidation, mitochondrial function disruption, and oxidative DNA damage (Fullston et al., 2017; Roychoudhury et al., 2017). Spermatozoa are particularly susceptible to ROS due to the presence of limited cytoplasmic antioxidants and restricted repair mechanisms (Attia et al., 2014). Owing to the translational and transcriptional inert nature of DNA, damage sustained in sperm DNA remains persistent (Attia et al., 2014). However, it is also important to consider that a multitude of risk factors have been postulated in relation to male infertility, encompassing erectile dysfunction, varicocele, congenital dysplasia, endo-crinological disorders, immunological aspects, sexually transmitted infections, and exposure to chemicals as well as radiation (Eisenberg and Lipshultz., 2011).

1.8 The impact of cannabis on sperm parameters

It is worth mentioning that, approximately 9% of daily tobacco smokers also smoke cannabis daily (Weinberger, A.H et al., 2020) and 40%–54% of cannabis users reported smoking cigarettes in the past 30 days (Pacek et al., 2018). Many studies have documented various reproductive anomalies, including compromised spermatogenesis, diminished semen quality, and modified sperm functionality in men who consume tobacco and cannabis (Martini et al., 2004; La Maestra e al., 2015).

The discovery of cannabinoid receptors on these cells implies that cannabinoids may affect the equilibrium of molecular signals that create this conducive environment. Notably, the CB2 receptor, located on Sertoli cells, has been identified as a critical player in inducing cell death, or apoptosis, in these cells. In addition, Leydig cells express the cannabinoid receptor CB1.

This receptor induces a reduction of testosterone production locally, which may impact sperm development (Duca, Y et al., 2019). In vitro studies have shown that THC has a negative impact on human sperm function. The detrimental effect on percentage progressive motility increases as overall semen quality declines. THC also reduces spontaneous acrosome reactions and inhibits the acrosome reaction, even at both therapeutic and recreational plasma levels (Rajanahally et al., 2019).

The interaction of cannabinoids with at least three enzymes involved in DNA repair has raised concerns regarding their potential genetic toxicity—a topic that has not yet been thoroughly studied. Toxicity can affect gonadal stem cells and genetics, leading to consequences such as inhibited cell growth, fetal deformities, and heritable defects, including cancers. Furthermore, there is evidence that cannabis use can result in altered DNA expression (Sarafian T et al., 2005). In a separate investigation conducted on male individuals aged 30 and under who consumed cannabis within a 90-day period before providing a semen sample exhibited a higher likelihood of abnormal sperm morphology (Pacey et al., 2014). The utilization of cannabis has been scientifically demonstrated to induce substantial morphological alterations in spermatozoa, manifesting as aberrant morphologies in experimental models with rodents such as mice and rats (Zimmerman et al., 1978).

In the study from Gundersen et al. regular use of marijuana was found to be associated with an impairment in semen quality, while irregular use seems to be irrelevant (Gundersen et al., 2015). Previous study conducted by Kolodny et al reported that 20 heterosexual men who had used marijuana at least four days a week for a minimum of six months experienced reduced plasma testosterone, oligospermia, and impotence (Kolodny et al., 1974).

The impact of cannabis on the motility of spermatozoa has been observed to decrease at both therapeutic and recreational levels of tetrahydrocannabinol (THC) (Whan et al., 2006). Numerous studies have documented various reproductive anomalies, including compromised spermatogenesis, diminished semen quality, and modified sperm functionality in men who consume tobacco and cannabis (Martini et al., 2004; La Maestra et al., 2015).

A recent study demonstrated that intense but short-term exposure to cannabis vapor lowered sperm counts and slowed spermatozoa motility, not only in the directly exposed male mice but also in their offspring (Shi, M.et al., 2022).

These findings, it is essential to note that prevailing research suggests that there is no association between cannabis use and chromosomal fragmentation within spermatozoa, implying to the preservation of genetic material (Generoso et al., 1985).

Also, RNA and DNA are perhaps the most critical biological targets affected by oxidative damage. Recent studies have linked oxidative harm in RNA molecules to a spectrum of neurological disorders, including Alzheimer's disease, Parkinson's disease, Down syndrome, dementia with Lewy bodies, prion disease, sub-acute sclerosing pan-encephalitis, and xeroderma pigmentosum. Among numerous types of RNA oxidative damage, the formation of 8-hydroxyguanosine (8-OHG) is a ubiquitous marker of oxidative stress (Sliwinska et al., 2016).

Researchers from Duke University (North Carolina) have demonstrated that cannabis consumption can modify the DNA methylation profile of exposed sperm (Schrott et al., 2020). They investigated the consequences of cannabis on human sperm, as well as the impact of tetrahydrocannabinol (THC), one of the primary active components of cannabis, on rat sperm. They employed reduced representation bisulfite sequencing (RRBS) to assay DNA methylation. Subsequently, they found that, in the case of human cannabis consumers, a marked reduction in sperm concentration was observed alongside alterations in 3,979 CpG sites exhibiting at least a 10% difference; predominantly leaning towards hypomethylation (Schrott et al., 2022). Nevertheless, an earlier study that investigated the offspring of rat parents subjected to THC exposure during adolescence found that these offspring exhibited modified brain DNA methylation profiles and a heightened tendency for self-administering heroin (Watson et al., 2015).

The CB1 receptors have demonstrated connections to mitochondrial activity within sperm, which is adversely influenced by cannabis exposure, culminating in compromised sperm motility. Although in vitro examinations have revealed plausible mechanisms, it remains uncertain whether these consequences are entirely mirrored in the male testicular environment (Barbonetti et al., 2010). The mitochondria, commonly acknowledged as the cellular powerhouse, possess their distinct genome encoding 13 proteins (Shamsi et al., 2008). The mitochondrial genome plays a pivotal role in mature sperm morphogenesis and flagellar motility following ejaculation (Nakada et al., 2006; Rajender S et al., 2010). Mutation rates within mitochondrial DNA (MT-DNA) are relatively high, attributed to the absence of histones and DNA repair mechanisms (Alexeyev et al., 2013). Consequently, mutations that arise within

the mitochondrial genome contribute significantly to particular human genetic disorders. It has been documented that MT-DNA mutations correlate with specific forms of male infertility, such as POLG locus mutations in MT-DNA polymerase (Rovio AT et al., 2001). Additionally, a high prevalence of single-nucleotide polymorphisms (SNPs) within MT-DNA has been observed in semen samples resulting in poor sperm quality (Holyoake AJ et al., 2001; Dahadhah et al., 2021; Al Smadi et al., 2021). Also, understanding the potential consequences of cigarette and cannabis smoking and their impact on male is crucial especially for men in reproductive age.

Therefore, this study is conducted to compare the effect of THC, CBD substances along with THC-COOH, the central secondary metabolite of THC generated within the body upon consumption of Cannabis, on sperm motility, chromatin integrity and DNA fragmentation of living sperm cells [In vitro study]. Secondly, to determine the impact of tobacco and cannabis smoking on sperm parameters, chromatin integrity, and oxidative damage as key indicators of male reproductive health. Finally, to determine the effects of tobacco and cannabis smoking in association with paternal mitochondrial genetic variants on spermatozoa functions. Namely, to find out the sperm mitochondrial genetic variants in the Cytochrome C Oxidase 1, 2 and 3 genes (MT-CO1, MT-CO2, and MT-CO3), investigate possible relationship with standard sperm parameters, protamination and spermatozoa DNA integrity.

2. MATERIAL AND METHODS

2.1 In vitro study of the effect of Cannabis on Spermatozoa

2.1.1 Study Design:

Semen samples were gained from male participants (n=10), aged 20 to 35 years, irrespective of their fertility status. Medical histories and health conditions were not considered in this prospective study, as its primary focus was to evaluate the quality of spermatozoa pre- and post- incubation with a specified toxic substance. All volunteers were formally requested, via written correspondence, to grant permission for their inclusion in this research study.

2.1.2 Sample Collection and Preparation:

Semen specimens were acquired via masturbation following a period of 2-7 days of sexual abstinence. These specimens were collected in sterile, wide, non-toxic receptacles and subsequently processed in the laboratory within 90 minutes post-ejaculation. Semen parameters, including volume, sperm count, and motility, were evaluated within two hours of collection in accordance with the 2021 WHO criteria (WHO 2021). A Computer Assisted Sperm Analysis (CASA) system was employed to ensure accurate results for the assessment of sperm parameters.

Multiple smears (10 µL) of each specimen were obtained to evaluate sperm chromatin integrity through the utilization of chromomycin CMA_3 and acridine orange assays.

Following this, 2 mL of each sample was exposed to 1 mL of a soluble variant of THC, CBD and THC-COOH supplied in a 95% methanol solution by LoGiCal Germany, at a concentration of 1 mg/mL

All samples were also exposed to 1 mL 95% methanol as a control group to eliminate the potential effects of methanol on sperm cells. The target compounds were introduced directly into the semen, allowing sperm to swim under normal conditions and preserving essential nutrients.

Upon completion of a one-hour incubation period at room temperature, sperm analyses were carried out for control and treated semen samples. Both control and *Cannabis* - treated samples underwent identical experimental procedures.

2.2 Impact of tobacco and cannabis consuming on sperm parameters and DNA quality

A total of 114 samples were collected from men aged less than 40 years at Prince Rashid bin Al Hassan Hospital (PRBH) in Irbid, Jordan and from IVF British-Syrian IVF center, Damascus, Syria.

Our participants' ages range between 25 to 40 years old, with all individuals hailing from West Asian backgrounds. We structured our participants into three distinct groups for this study:

1. Group 1'NS' consists of non-smokers, non-Cannabis who serve as the control group, with a total number of 38 individuals. Control group participants have no history of tobacco or cannabis use.

2. Group 2'TS' includes tobacco smokers but non-cannabis (n= 39). These individuals have been smoking more than 10 cigarettes daily for at least five years. Participants in tobacco smoker group have exclusively used tobacco and have never used cannabis or any other recreational drug.

3. Group 3 'CS' encompasses cannabis smokers (n= 37) participants. Each participant in this group has been using cannabis for more than three years, consuming at least four joints per week. Most participants within our cannabis smoker group are also current tobacco users or at least have smoked tobacco in the past.

All men were potential candidates for providing sperm samples pertinent to our research. To initiate participation, we developed a comprehensive questionnaire designed to ascertain whether individuals would meet the rigorous criteria established for our study.

Participants were then carefully selected based upon their responses, placing particular emphasis on lifestyle habits such as smoking; individuals were grouped accordingly based on their smoking habits. This allowed us to examine any variations attributed to such behaviors within the dataset. Moreover, patients older than 40 years old consuming alcohol daily, or having diabetes mellitus, chronic disease, recent infection and genetic abnormalities such as Klinefelter's syndrome were excluded from the study. In addition, patients with alcoholic problems, and genetic abnormalities were excluded from the study.

Semen samples were collected from all subjects by masturbation after 3 to 5 days of sexual restraint. Thereafter, semen analysis was done within 30 minutes to 1 hour of ejaculation also

following a period of incubation at 37°C to allow for liquefaction according to WHO recommendations (WHO. 2021).

Following measurement of semen volume, samples were analyzed using standard light microscopy within one hour of ejaculation. Several 10 µL smears of each sample were done to evaluate spermatozoa morphology, chromatin integrity using chromomycin (CMA3), DNA strand breaks by acridine orange assays (AO).

Each donor was: a) in good physical and mental health, b) free from any hereditary diseases, c) tested negative for HIV types 1 and 2, syphilis, hepatitis B and C, herpes, and cytomegalovirus, d) showing no bacterial infections in blood or semen cultures, and e) possessing a seminal profile that surpasses the minimum standards set by the World Health Organization guidelines (WHO 2021). Semen analysis, sperm chromatin condensation integrity with Chromomycin (CMA_3) staining, sperm viability, DNA fragmentation (Acridine Orange) and DNA-RNA oxidative damage were compared between the study groups.

2.2.1 Chromatin condensation (Chromomycin CMA_3):

Chromatin integrity in human sperm was evaluated by Chromomycin (CMA_3) staining method. Briefly, the air-dried semen smears were fixed in methanol-glacial acetic acid 3:1 at 4°C for 60 minutes and then allowed to air dried at room temperature. 50 µL of CMA_3 stain solution (0.25 mg CMA_3/mL in McIlvain's buffer PH 7.0, supplemented with10 mmol/L Mg Cl_2) was added to each slide and covered with coverslips then incubated in the dark for 30 minutes at room temperature.

Lastly, each slide was rinsed with PBS buffer and mounted with 1:1 (v/v) PBS/glycerol then kept at 4°C overnight. A total of 200 spermatozoa were observed on each slide under fluorescent microscope with a 460-nm filter and 10 eye piece magnifications. The assessment of chromatin condensation states involved differentiating between spermatozoa exhibiting bright yellow stains (CMA_3 positive) and those displaying dull yellow stains (CMA_3 negative), as depicted in Figure 10 (Talebi et al., 2008).

2.2.2 Acridine Orange (AO):

The analysis of DNA fragmentation in spermatozoa was evaluated by using Acridine Orange fluorescence staining. This staining was performed according to the methods described thoroughly by Hammadeh et al., (2010). Briefly. 1. air-dried semen smears were fixed for 2 hours in freshly prepared Carnoy's solution (Methanol/glacial acetic acid 3:1), then air dried

2. Smears were stained with acid Acridine Orange solution

3. All smears were prepared for assessment using fluorescence microscope. For this study, each slide was carefully examined to analyze 200 sperm cells. Acridine Orange (AO), a fluorescent dye, was used to differentiate the DNA integrity within these cells. AO intercalates with double-stranded DNA; however, in sperm cells with immature nuclei, DNA is readily denatured into single strands. This results in an aggregation of AO molecules within the nuclei, emitting an orange-red fluorescence. Conversely, sperm nuclei with intact double-stranded DNA exhibit green fluorescence. This distinction allows for the identification of spermatozoa with denatured (orange or yellow, AO positive) and normal (green, AO negative) DNA (Varghese, Alex C., 2011). Observations were carried out using a BX51 fluorescence microscope from Olympus Corporation equipped with a 480–490 nm filter.

The prepared slides were subsequently examined under a fluorescence microscope for analysis. The percentages of spermatozoa displaying green fluorescence, indicating normal DNA integrity, and orange-red fluorescence, which signals abnormal DNA integrity, were then calculated. In order to thoroughly evaluate the samples, a total of 200 spermatozoa were scrutinized from each slide, categorizing them based on their staining characteristics into either orange or yellow (acridine orange positive, indicative of denatured DNA) or green (acridine orange negative, representative of double-stranded and normal DNA) (Hammadeh et al., 2010).

2.2.3 ROS measurements:

The Oxidative DNA-RNA oxidative damage evaluated by applying Enzyme-Linked Immunosorbent Assay (ELISA) kit

The Oxidative RNA Damage Enzyme-Linked Immunosorbent Assay (ELISA) kit is competitive immunoassay for the accurate quantification of 8-hydroxyguanosine (8-OHG) (Cell biolabs company). Initially, the 8-OHG samples of unknown concentrations or established 8-OHG standards are incorporated into a pre-absorbed EIA plate featuring an 8-OHG/BSA conjugate. Following a brief incubation period, a highly specific anti-8-OHG monoclonal antibody is introduced, succeeded by the addition of a horseradish peroxidase (HRP)-conjugated secondary antibody. The precise 8-OHG content in the unknown samples was ascertained through comparison to a predetermined 8-OHG reference curve (Sliwinska et al., 2016).

Table 2; Preparation of 8-OHG Standards

Standard Tubes	8-OHG Standard (µL)	Assay Diluent (µL)	8-OHG (ng/mL)
1	20	980	40
2	500 of Tube #1	500	20
3	500 of Tube #2	500	10
4	500 of Tube #3	500	5
5	500 of Tube #4	500	2.5
6	500 of Tube #5	500	1.25
7	500 of Tube #6	500	0.625
8	500 of Tube #7	500	0.313
9	500 of Tube #8	500	0.156
10	0	500	0

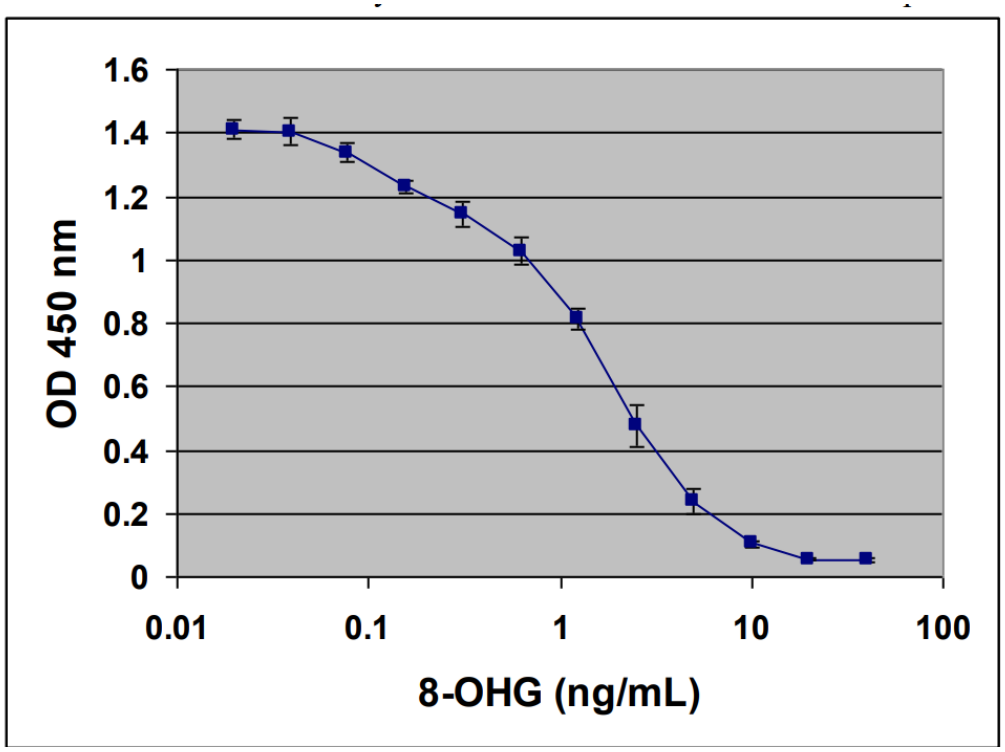


Figure 7; 8-OHG Elisa Standard Curve.

2.3 Mitochondrial study: Ssubjects and study design

One hundred and thirteen semen samples were collected from men of reproductive age attending in vitro fertilization labor (IVF) at Prince Rashid Bin Al Hassan Hospital (PRBH) in Irbid, Jordan.

Patients who smoked more than one pack per day for 10 years were considered as Tobacco-smokers. Patients who smoked at least 4 joints in a week for more than 3 years were considered as cannabis smokers. Thus, we had three groups: Non-smokers (N=37), Tobacco-smokers (N=39), and Cannabis-smokers (N=37).

2.3.1 DNA extraction:

To isolate total DNA and mitochondrial DNA, frozen semen samples were first defrosted and refined employing 40%–80% Puresperm discontinuous gradients (Nidacon International,

Sweden), to eliminate non-sperm cells. Samples were arranged atop pre-prepared density gradient tubes and subjected to centrifugation at 500 g for 20 minutes at room temperature.

DNA extraction was executed using QIAamp DNA Mini Kit by QIAGEN, commonly applied for both genomic and mitochondrial DNA isolation. According to the method described in the accompanying handbook (Figure 8). Initially, a 20-microliter dose of Protease K enzyme was dispensed into a 1.5 ml microcentrifuge tube. Subsequently, 200 microliters of washed semen sample were introduced to the same tube and mixed well. The mixture was then incubated at 56°C overnight to maximize DNA extraction.

Next, 200 microliters of Buffer AL were incorporated into the mixture and homogenized by vortexing for 15 seconds. Then, an equal volume of absolute ethanol (96–100%) was added, followed by another vortex cycle for 15 seconds. To discard any liquid from the tube cap interiors, a brief centrifugal pulse was applied. The resultant blend was then poured into a QIAamp Mini spin column placed within a 2 ml collection tube and centrifuged at 8000 rpm for one minute.

Subsequently, the spin column was transferred to a fresh collection tube supplied with the kit. The filtrate-containing tube was disposed of carefully. All spin columns were sealed during spins to prevent aerosol dispersal. After opening each column, 500 microliters of Buffer AW1 were added followed by centrifugation at the previously noted speed and duration.

The process continued with another transfer of the spin column into a new collection tube where it received an additional cycle using 500 microliters of Buffer AW2 at maximum speed (20,000 g or 14,000 rpm) for three minutes.

Finally, the spin columns were relocated to new 1.5 ml microcentrifuge tubes, discarding those holding filtrates. A measure of 200 microliters of Buffer AE or distilled water was added. To enhance DNA retrieval, then incubated at room temperature (ranging from 15°C to 25°C) for one minute before being centrifuged at 6000 x g for one minute to complete the process.

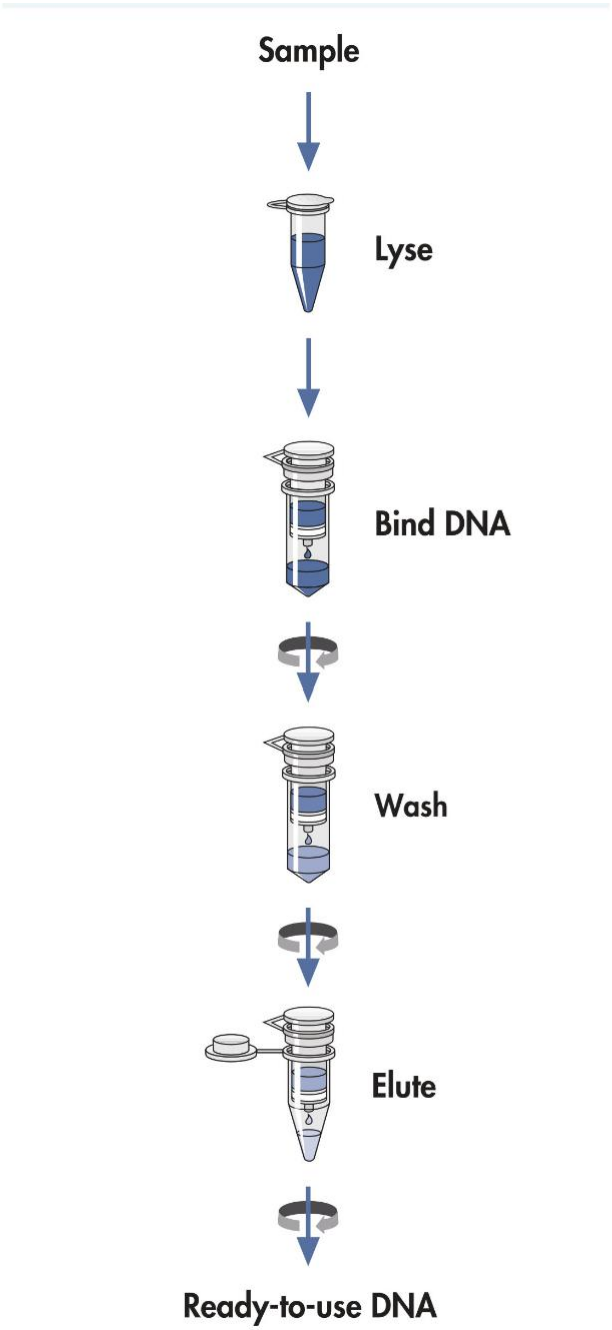


Figure 8; procedure of QIAamp DNA Kits for isolation of genomic DNA (Handbook, B. D. P. (2005). Qiagen. Gmbh, Germany)

2.3.2 MT-DNA amplification:

In the second phase of the procedure, mitochondrial DNA was selectively amplified using QIAGEN's REPLI-g Mitochondrial DNA Kit, in strict conformity with the protocol and recommendations provided with the kit. The process began by dispensing 10 µl of the extracted DNA sample into a microcentrifuge tube, followed by the addition of RNase-Free water to increase the volume to 20 µl. Subsequently, 29 µl of a freshly prepared amplification mixture (27 µl REPLI-g mt Reaction Buffer + 2 µl REPLI-g Human mt Primer Mix), was transferred to the DNA sample tubes using pipette action, which was then subjected to vortexing and another round of centrifugation. The reaction mix underwent incubation at 75°C for 5 minutes in a thermocycler and was allowed to gradually return to room temperature. This step was succeeded by adding 1 µl of REPLI-g Midi Polymerase to the DNA preparation and incubating it for 8 hours at 33°C.

The enzyme was subsequently deactivated by heating the samples at 65°C for 3 minutes. This step was followed by quantifying the DNA concentration in the isolated samples via the NanoDrop spectrophotometer, selecting only those with an absorbance ratio (260/280 nm) of at least 1.8 as optimal. Lastly, the resultant mitochondrial DNA solutions were stored at -80 ºC for future use in PCR analyses.

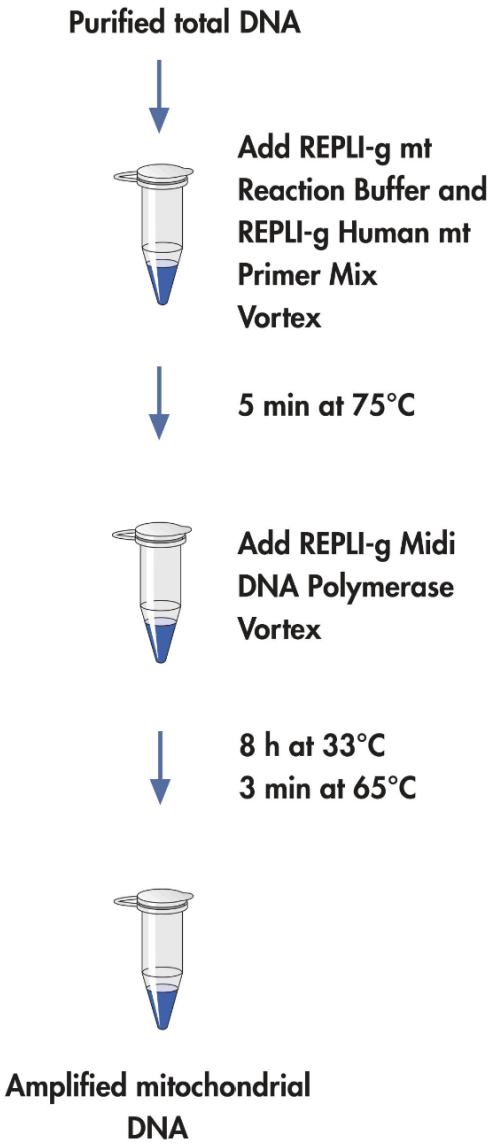


Figure 9; Amplification of mitochondrial DNA using the REPLI-g Mitochondrial DNA Kit

2.3.3 Polymerase chain reaction (PCR):

Table 3; Primers list for PCR amplification and Sanger sequencing

Primer name	Sequence (5′– 3′)	Product length
Mt-Co1 Forward	TCA CCC CCA CTG ATG TTC G	1542 bp
Mt-Co1 Reverse	GGG GGT TCG ATT CCT TCC TTT T	1542 bp
Mt-Co2 Forward	ATA TCT TAA TGG CAC ATG CAG C	684 bp
Mt-Co2 Reverse	GAG GGG GTG CTA TAG GGT AA	684 bp
Mt-Co3 Forward	GCA CGA CAA CAC ATA ATG ACC C	784 bp
Mt-Co3 Reverse	ACT AAA AGA GTA AGA CCC TCA TCA	784 bp
Mt-Co1 Forward Plus*	TTTACAGTAGGAATAGACGTA	*
Mt-Co1 Reverse Plus*	ACCGAAAAATCAGAATAGGTG	*

*** Additional internal primers were designed for Sanger sequencing only.**

To amplify the mitochondrial genes MT-CO1, MT-CO2, and MT-CO3, three sets of polymerase chain reaction (PCR) primers (forward and reverse) were meticulously designed utilizing the Primer 3 software. These primers were designed based on the human mitochondrial sequence procured from the National Centre of Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov). The oligonucleotide primers were synthesized by Microsynth Seqlab in Germany, as detailed in Table 3.

A 25 µL reaction mixture was prepared, comprising 12.5 µL PCR Master Mix (2X) (Thermo Scientific), 0.8 µL of 10 mM forward primer, 0.8 µL of 10 mM reverse primer, 2 µL MT-DNA (20 ng/µL), and 8.9 µL nuclease-free water. The Thermocycler (C1000™ Thermal cycler, Bio-Rad, USA) was used following this program: an initial denaturation at 95 °C for 3 min ensued by 35 cycles of denaturation at 95 °C for 30 s. Then, annealing step for 40 s (Mt-Co1: 59 °C; Mt-Co2 and Mt-Co3: 61°C), extension of primers at 72 °C for 1 min, and a final extension for 5 min at 72 °C.

To verify amplification, a subsequent analysis was conducted by running a 5 µL aliquot of each PCR product on a 1% agarose gel stained with GelRed® Nucleic Acid Stain. Visualization was achieved using Molecular Imager Gel Doc XR+ (Bio-Rad, USA).

2.3.4 Detection of variants in cytochrome c oxidase 1, 2 and 3 (MT-CO1, MT-CO2, and MT-CO3):

The products of PCR sent for purification and analyse via Sanger sequencing at a local company Microsynth Seqlab, Germany. A bidirectional sequencing (forward and reverse) was conducted for each specimen. Concerning the MT-CO1 gene, two supplementary internal primers were designed for sequencing (Table 3).

Primary and secondary sequences of every sample were analysed using the Mutation surveyor (Version 5.2.0), BioEdit sequence alignment editor version 7.2.5, and Unipro UGENE (Version 50.0) software.

3. Statistical Analyses:

Data analysis was conducted using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). Statistical calculation and graphs were generated using GraphPad Prism 6 software. Data with normal distribution are presented as mean ± standard deviation. The Shapiro-Wilk test determined the normality of data distribution.

The Kruskal-Wallis H test was employed to discern statistically significant differences in sperm parameters, AO, CMA3, and 8-OHG RNA damage content among the three groups Non-smokers, smokers, and cannabis smokers.

The differences between groups were calculated using the t-test for means, Chi-square, and Fisher exact test for the non-numerical variables. Data are represented as Mean ± SEM (standard error of the mean). The comparisons between the three groups were made using one-way analysis of variance (ANOVA). Conversely, for non-normally distributed variables, the Mann-Whitney U test was applied as appropriate. The p-value <0.05 was considered as statistically significant and $p < 0.01$ as highly significant.

3.1 Ethical issues:

A written informed consent from each participation in the study was obtained. All procedures were approved by each site's institutional review board. The study was approved by the Jordanian Royal Medical Services-Human Research Ethics Committee, with the project identification code (TF3/1/Ethics Committee/9126).

4. Results:

4.1 Results of the In vitro study of the effect of Cannabis on Spermatozoa

The clinical data of semen and sperm analysis of all participants (n=10) as described in Table 4. The median of total sperm motility, progressive motility and immotile sperms were (68.7%, 24.4% and 31.3% respectively).

By introducing just 10 µL of THC, THC-COOH, and CBD to sperm cells, a significant reduction in their overall motility was observed. Both THC and CBD had a remarkable impact on sperm motility, with statistical significance values of $P \leq 0.001$ and $P \leq 0.003$ respectively. Conversely, only a slight inhibition of total sperm motility was noted following incubation with THC-COOH. Intriguingly, the quantity of immotile sperm significantly increased to the maximum when treated with THC and CBD ($P \leq 0.001$ and $P \leq 0.003$, respectively). Similar trends were detected in relation to progressive motility (THC: $P \leq 0.001$ and CBD: $P \leq 0.004$) (Figure 11).

No discernible alterations were identified in either morphology or chromatin's integrity of the sperm cells after a one-hour incubation period with the studied substances. This finding aligns with expectations, as modifications to chromatin and nuclear material typically transpire through gradual processes rather than occurring instantaneously.

Table 4; Descriptive Characteristic and Clinical Data of All Participants (n=10) Before Incubation

	Minimum	Maximum	Mean	SD
Sperm count (Million/mL)	30	110	67.5	27
Total sperm motility %	31	80	68.7	15.12
Sperm progressive motility %	2	47	24.4	14.53

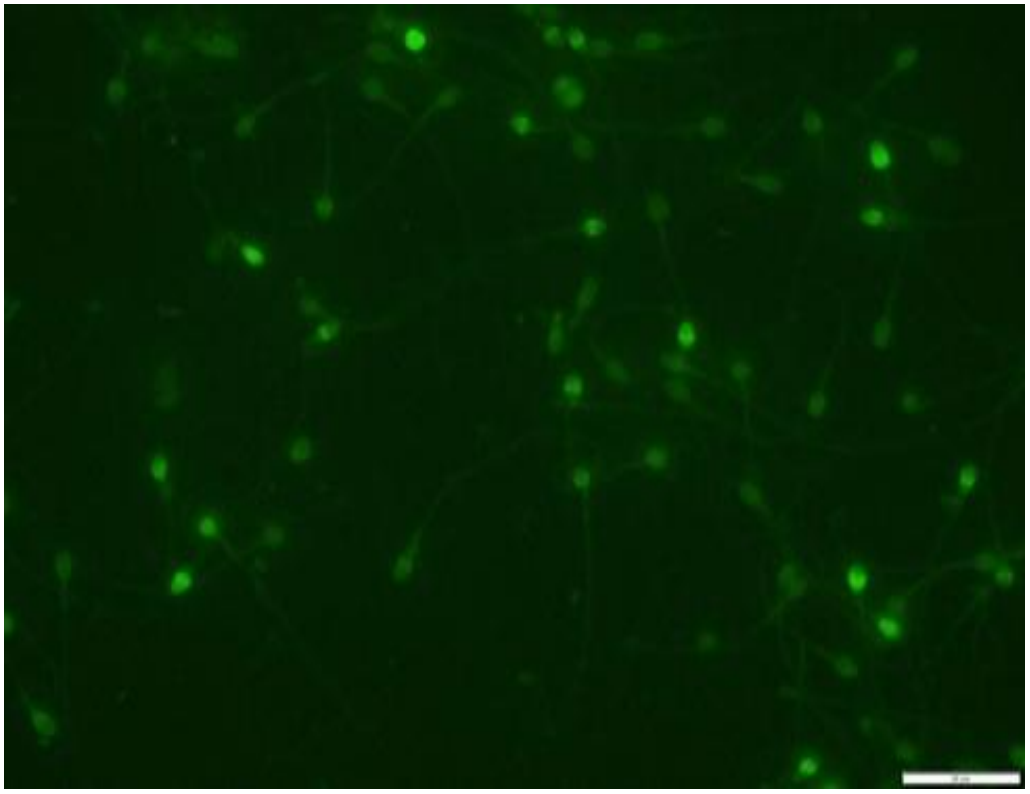


Figure 10; Self-image of Chromomycin A3 (CMA3) staining. Spermatozoa exhibiting positive chromomycin A3 (CMA3) staining are characterized by a bright appearance, while those with negative CMA3 staining display a dull appearance.

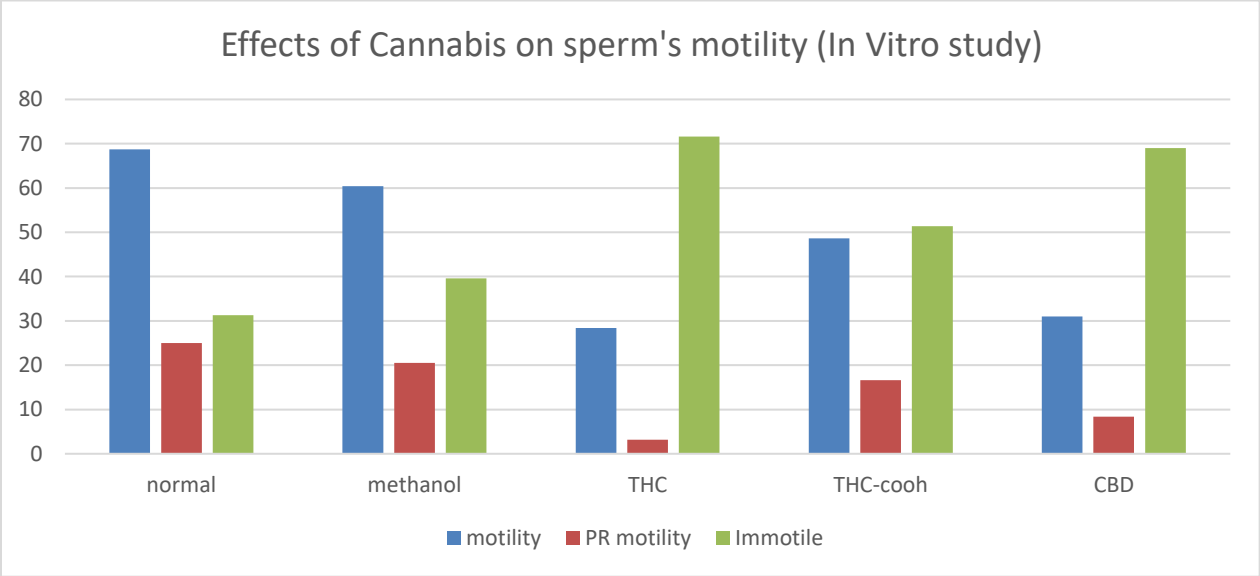


Figure 11; Results of Sperm motility after 1h of incubation with THC, THC_COOH and CBD, in the absence of any substances (normal) and the presence of just methanol 95% (methanol) as control to exclude its effect alone on sperm motility. As the other substances were dissolved in methanol.

4.2 Smoking (Tobacco and Cannabis) and its correlation to sperm parameters and DNA quality

The study population included three groups: non-smoker individuals (NS, N=37), tobacco smoker individuals (TS, N=39), and cannabis smoker individuals (CS, N=37).

The semen analysis included sperm morphology, volume, motility, and count. In addition, AO, and CMA3 tests were performed in the three study groups.

Routine semen parameters in the cigarette (Tables 5) and cannabis smoker's groups (Tables 6) were compared with those of the non-smoker's group.

Sperm counts was (59.64 ±23.9) mill /mL for non-smokers, 57.92 ±21.6 mill /mL for smokers and 46.71 ± 18.1 mill /mL for cannabis smokers ($p = 0.159$). Progressive motility was 57.02 ± 11.15 % for non-smokers, 60.77 ±10.5 % for smokers, and 46.21 % for cannabis smokers, $p = 0 .110$).

Although the sperm count and progressive motility exhibited minor reductions, also, neither the counts nor the motility showed statistically significant differences between the three investigated groups Tab.3 and 4). In both groups, cigarette or cannabis smokers, no significant differences in sperm concentration or sperm motility were found between the smoking and non-smoking groups.

On the contrary, the findings revealed a statistically significant discrepancy in the immotile sperm count across distinct smoking categories. A notable variation was observed in the quantity of immotile sperms and sperms exhibiting abnormal morphology among the groups studied.

The immotile spermatozoa demonstrated a statistically significant increase in smokers and cannabis smokers in comparison to non –smokers (44.59±18.5 mill/mL) for non-smokers; 52.42±24.30mill/mL for smokers, and 65.13±21.8mill/mL) for cannabis smokers $p \leq 0.020$) Figure 12.

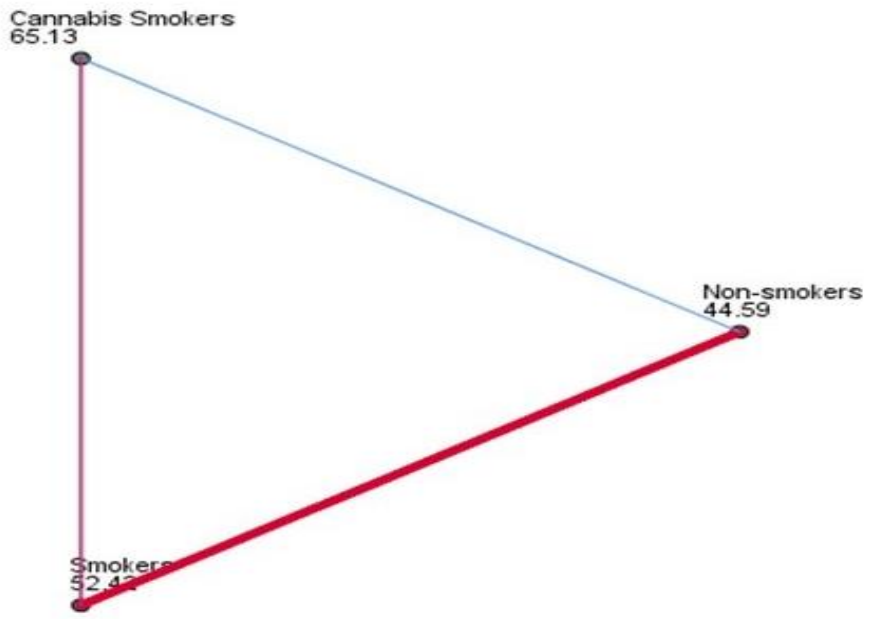


Figure 12; Pairwise comparisons of immotile sperms. Each node shows the sample average rank of each group.

There was virtually no statistically significant difference between non-smoker and tobacco smoker groups. However, a significant difference in immotile sperm was observed exclusively between the non-smoker and cannabis smoker group. The difference between tobacco smoker group and cannabis smoker groups less pronounced (Tab. 2).

Concurrently, the mean number of morphologically normal spermatozoa exhibited a significant decline as a follow (7.34± 5.8 % for non-smokers, 5.2.02±4.8 % for smokers, and 2.232±2.1 % for cannabis smokers' $p \leq 0.001$) Figure 13.

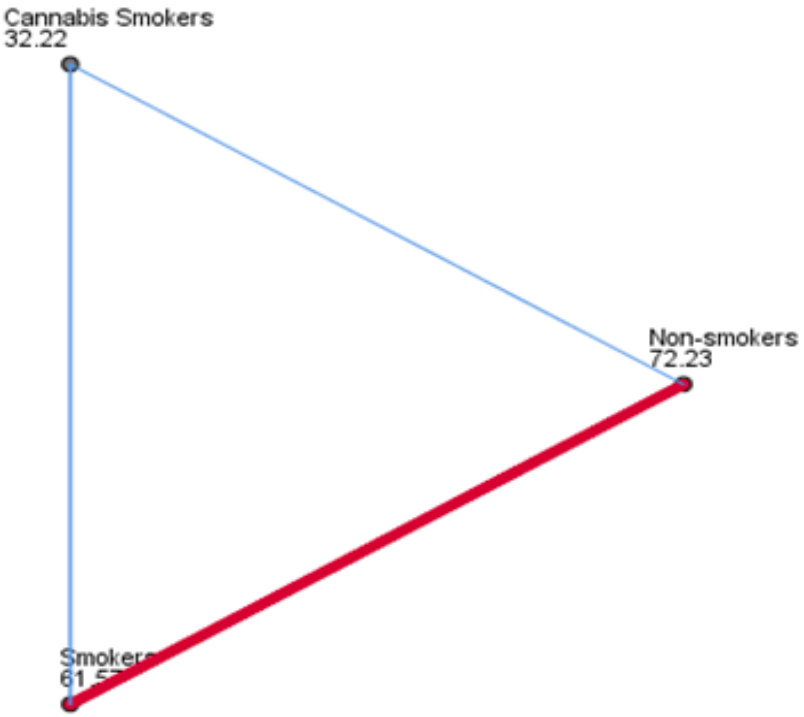


Figure 13; Pairwise comparisons of sperm's morphology. Each node shows the sample average rank of each group.

Also, no discernible differences were found between tobacco smokers and non-smokers groups ($P \leq 0$ .152). However, a statistically significant difference ($P \leq 0.001$) was identified between the cannabis smoker group and both non-smoker and tobacco smoker groups.

The mean percentage of Chromatin condensation CMA3 was (14.68±15.64 % for non-smokers 25.28±14.86% for tobacco smokers and 34.83±18.39% cannabis smokers).

The percentage of abnormal sperm chromatin condensation was significantly higher in smokers compared to nonsmokers ($p \leq 0.001$).

A statistically significant difference in mean values was observed between the investigated groups ($p \leq 0.001$). A post hoc analysis using the Tukey HSD- test indicated that non-smokers exhibited considerably lower CMA3+ scores compared to both tobacco and cannabis smokers.

The mean percentage of spermatozoa stained positively with Acridine Orange (DNA damage) was (10.08±14.2 % for non-smokers, 16.5±10.2 % for tobacco smokers, and 28.5± 15.8 % for

cannabis smokers. However, no significant difference was observed in the mean percentage of AO positive stained samples (DNA damage) among the groups ($p \leq 0.481$)

Furthermore, the mean concentrations of DNA-RNA Oxidative Damage were 40.74±18.2 for non-smokers, 46.26±19.30 for tobacco smokers, and 88.42± 22.40 for cannabis smokers (Tables 5, 6, 7).

The proportion of oxidative damage in the cannabis smoker cohort was significantly elevated in comparison to both non-smoker and tobacco smoker groups ($p \leq 0.001$). (Table 6, 7).

Table 5; Comparison of semen parameters between cigarette smokers and non-smokers.

Parameters	**Non-smokers "NS"**	**Tobacco Smoker "TS"**	**P-Value**
Sperm concentration (mi/mL)	59.64 ± 23.9	57.92 ± 21.6	$p \leq 0.487$
Sperm Motility (%)	57.02± 11.15	60.7± 10.5	$p \leq 0$ .399
Immotile sperm (%)	44.59± 18.5	52.42± 24.3	$p \leq 0.296$
Sperm morphology	7.34± 5.8	5.02± 4.8	$p \leq 0.152$
Chromatin condensation (%) (CMA3)	14.68±15.64	25.28±14.86	$p \leq 0.024$
DNA damage (Acridine Orange AO)	10.08± 14.2	16.5± 10.2	$p \leq 0.499$
DNA-RNA Oxidative Damage (%)	40.74± 18.2	46.26± 19.3	$p \leq 0.464$

Table 6; Comparison of semen parameters between cannabis smokers and non-smokers.

Parameters	**Non-smokers "NS"**	**Cannabis smoking "CS"**	**P-Value**
Sperm concentration (mi/mL)	59.64 ± 23.9	46.71 ± 18.1	$p \leq 0.159$
Sperm Motility (%)	57.02± 11.15	46.21± 10.5	$p \leq 0.114$
Immotile sperm (%)	44.59± 18.5	65.13± 21.8	$p \leq 0.006$
Sperm morphology	7.34± 5.8	2.23± 2.1	$p \leq 0.001$
Chromatin condensation (%) (CMA3)	14.68±15.64	34.83±18.39	$p \leq 0.001$
DNA damage (Acridine Orange AO)	10.08± 14.2	28.5±15.8	$p \leq 0.481$
DNA-RNA Oxidative Damage (%)	40.74± 18.2	88.42± 22.4	$p \leq 0.001$

Table 7; Comparison of semen parameters between cigarette smokers and cannabis smokers.

Parameters	**Tobacco Smoker "TS"**	**Cannabis Smoker "CS"**	**P-Value**
Sperm concentration (mi/mL)	57.92 ± 21.6	46.71 ± 18.1	$p \leq 0.168$
Sperm Motility (%)	60.7± 10.5	46.21± 10.5	$p \leq 0.110$
Immotile sperm (%)	52.42± 24.3	65.13± 21.8	$p \leq 0.791$
Sperm morphology	5.02± 4.8	2.23± 2.1	$p \leq 0.001$
Chromatin condensation (%) (CMA3)	25.28±14.86	34.83±18.39	$p \leq 0.025$
DNA damage (Acridine Orange AO)	16.5± 10.2	28.5± 15.8	$p \leq 0.481$
DNA-RNA Oxidative Damage (%)	46.26± 19.3	88.42± 22.4	$p \leq 0.001$

Upon utilizing the one-way ANOVA technique to analyze data collected from the groups (NS, TS, CS), we observed statistically significant differences across a broad spectrum of parameters under examination, including morphology, motility, AO+, CMa3+ staining, and the extent of DNA-RNA oxidative damage. The p-values associated with these findings were all less than 0.001, indicating a high level of statistical significance in the variations noted between the groups for each measured parameter (Table 5).

The results showed a significant reduction in normal sperm morphology in tobacco smokers (5, 02 ± 4.8%) and cannabis smokers (2,26 ± 2.3%) groups compared to the non-smoker group (7,46 ± 5.9) ($p < 0.001$) (Table 5). Moreover, there was a significant reduction in normal sperm morphology in the cannabis smoker group compared to the tobacco smoker group ($p = 0.002$) as shown in Figure 14.

At the same time, there was no significant difference in sperm count between the tobacco-smoker (30,65 ± 21.6 x 10^6) and cannabis-smoker groups and non-smoker group ($p = 0.199$) as shown in Figure 15. In addition, semen volume did not show significant differences between groups ($p=0.091$) as shown in Table 5.

Table 8; Comparison of the semen parameters between non-smokers, tobacco-smokers and Cannabis smoker groups, one way ANOVA.

Parameters	NS **(Mean±SD)**	TS **(Mean±SD)**	CS **(Mean±SD)**	One Way-ANOVA P-Value
Age (Years)	35.13 (± 7.7)	32.95 (±6.3)	28.05 (± 3.7)	0.061
Volume (ml)	3.37 (± 1.2)	3.47 (± 1.4)	3.05 (± 0.8)	0.091
Count (10^6/ml)	33.86 (± 24.1)	30.65 (± 21.6)	28.37 (± 18.2)	0.199
Morphology (%)	7.46 (± 5.9)	5.02 (± 4.8)	2.26 (± 2.3)	**< 0.001**
PR Motility (%)	14.27 (± 11.3)	13.12 (± 10.6)	10.18 (± 10.6)	0.223
NP motility (%)	34.40 (± 14.3)	27.82 (± 16.6)	20.63 (± 12.6)	**< 0.001**
Immotile (%)	51.73 (± 18.8)	58.92 (± 24.4)	68.66 (± 21.9)	**< 0.001**

Sperm progressive motility showed a non-significant reduction in the cannabis-smoking group (10.18 ± 10.6%) compared to tobacco smoking (13.12 ± 10.6%) and the non-smoking group (14.27 ± 11.3%) (p=0.223) (Table 8). Moreover, the mean percentage of non-progressive motile spermatozoa demonstrated a significant reduction in the cannabis-smoking group (20.63 ± 12.6%) and the tobacco-smoking group (27.82 ± 16.6%) in comparison to the non-smoking group (34.40 ± 14.3%) ($p < 0.001$).

However, the mean percentage of immotile sperm was significantly higher in the cannabis-smoking group (68.66 ± 21.9%) compared to tobacco smoking (58.92 ± 24.4) and the non-smoking group (51.73 ± 18.8 %) ($p < 0.001$) (Table 8).

Table 9; Comparison of sperm DNA fragmentation assessed by AO staining (AO+) and the grade of protamine deficiency in sperm DNA assessed by Chromomycine-A3 staining (CMA3+) between NS, TS, and CS, one way ANOVA.

Parameters	Non-Smokers **(Mean ± SD)**	Tobacco smokers **(Mean ± SD)**	Cannabis Smokes **(Mean ± SD)**	One Way-ANOVA P-Value
AO+ (%)	10.1 ±14.2	6.4 ±10.2	28.53 ± 15.8	**< 0.001**
CMA3+ (%)	15.0 ± 15.4	25.3 ±14.9	37.13 ± 20.1	**< 0.001**

DNA integrity was measured using an Acridine Orange (AO) assay and chromomycine Staining (CMA_3). The results showed a significant increase in the AO+ score in the cannabis-smoking group (28.53 ± 15.8%) compared to the non-smoking group (10.1 ± 14.2%) and the tobacco-smoking group (6.4 ±10.2%) ($p < 0.001$) (Table 9). At the same time, there was no significant difference in the AO+ score between the tobacco-smoking and non-smoking groups ($p = 0.19$) as shown in Figure 16.

In addition, the CMA3+ test showed a significant increase in the CMA_3+ score in the cannabis-smoking group compared to the non-smoking group ($p < 0.001$) and between the tobacco-smoking group compared to the non-smoking group. At the same time, CMA_3+ score was significantly higher in cannabis-smoking men in comparison to tobacco-smoking men ($p = 0.001$) as shown in Figure 17.

Furthermore, the mean concentrations of DNA-RNA Oxidative Damage were 40.74 % ±18.2 for non-smokers, 46.26 % ±19.30 for tobacco smokers, and 88.42± 22.40 for cannabis smokers.

The proportion of oxidative damage in the cannabis smoker cohort was significantly elevated in comparison to both non-smoker and tobacco smoker groups ($p \leq 0.001$)

4.3 MT-CO1, MT-CO2 and MT-CO3 SNPs distribution between non-smokers, tobacco smokers and Cannabis Smokers

In the mitochondrial cytochrome c oxidase subunit 1 (Mt-Co1) gene, a cumulative sum of 22 single nucleotide polymorphisms (SNPs) was detected. With a distribution of 37,8 %, 64,1 %, and 54 % among non-smoker, tobacco smoker, and cannabis smoker groups, respectively. Among these variations, eight were classified as missense mutations (rs1556423059, rs1603220225, rs200165736, rs201262114, rs1603220429, rs879164161, rs1556423267 and rs200784106). And the other Fourteen as synonymous substitutions (rs1603220215, rs879112886, rs879104796, rs2124593224, rs1556423086, rs1029272, rs878870695, rs879050330, rs386420010, rs370472320, rs386829005, rs201395766, rs879118820 and rs2015062), as delineated in Table 10.

Among the observed aberrations, "Two single nucleotide polymorphisms (SNPs) were identified with increased frequency among groups. Specifically, rs1556423086 demonstrated a

marked distinction, wherein nucleotide C replaces T at position chrMT:6251. Nevertheless, this alteration did not affect the resulting amino acid, maintaining Valine -> Valine (synonymous variant). This particular variation was clearly more prevalent in the cannabis smokers' group.

The other variant identified was rs201262114, located at the chromosomal position chrMT:6261. This particular mutation, which has been markedly observed in the tobacco smoker's cohort compared to other groups, constitutes a missense variant that leads to an amino acid substitution of Alanine to Threonine.

On the other hand, 17 Single Nucleotide Polymorphisms (SNPs) have been identified within the mitochondrially-encoded Cytochrome C Oxidase II (MT-CO2) gene, with a proportion of 21,62 %, 28,2 %, and 43,24 % among non-smoker, tobacco smoker, and cannabis smoker groups, respectively. Five of these mutations were categorized as missense variants, involving single nucleotide alterations that led to alterations in the amino acid sequence: rs1603221063, rs1556423339, rs879119797, rs386420037 and rs878897170. The remaining Twelve variants were classified as synonymous: rs1556423316, rs1556423319, rs1603221066, rs1556423330, rs1603221136, rs386829014, rs1603221150, rs368038563, rs879161183, rs2068706923, rs1603221307 and rs3021089. Table 11

In the mitochondrial-encoded cytochrome c oxidase III gene (Mt-Co3), a total of 20 single nucleotide polymorphisms (SNPs) were identified, with a distribution of 54,05 %, 64,1 %, and 51,35 % among the investigated groups - non-smokers, tobacco smokers, and cannabis smokers, respectively. Among these SNPs, only Four were missense variants: rs1556423681, rs1556423714, and rs1556423726, and rs1603222339. The other Thirteen mutations rs9743, rs386829084, rs2248727, rs1556423706, rs374335946, rs1603222253, rs2853824, rs2856985, rs375478739, rs1603222347, rs879070193, rs386829074 and rs879229894, were synonymous. Additionally, our findings included Three SNPs that were not listed in the NCBI database. Among these, two were Synonymous Variants located at positions 9657 and 9758 in the mt-co3 gene, and one was a Missense Variant found at chromosome position 9689. Table 12

Table 10; Genotype frequencies of cytochrome oxidase 1 gene (MT-CO1) variants in non-smokers, tobacco-smokers, and Cannabis-smokers' cases

SNPs ID	Position	Mutation type	Amino acid change	Genotype	NS (N=37)	TS (N=40)	CS (N=38)
rs1603220215	m.5996 A>G	Synonymous Variant	Thr31	AA	37	39	35
				AG	0	0	0
				GG	0	0	2
rs1556423086	m.6152 T>A	Synonymous Variant	Val83	TT	37	39	33
				TA	0	0	0
				AA	0	0	4
rs1029272	m.6185 T>C	Synonymous Variant	Phe94	TT	36	36	37
				TC	0	0	0
				CC	1	3	0
rs879112886	m.6026 G>A	Synonymous Variant	Leu41	GG	36	39	36
				GA	0	0	0
				AA	1	0	1
rs879050330	m.6518 C>T	Synonymous Variant	Gly205	CC	37	38	34
				CT	0	0	0
				TT	0	1	3
rs386420010	m.6446 G>A	Synonymous Variant	Thr181	GG	34	39	37
				GA	0	0	0
				AA	3	0	0
rs370472320	m.6221 T>C	Synonymous Variant	Pro106	TT	37	38	37
				TC	0	0	0
				CC	0	1	0
rs386829005	m.7337 G>A	Synonymous Variant	Ser478	GG	35	38	37
				GA	0	0	0
				AA	2	1	0

rs2015062	m.7028 C>T	Synonymous Variant	Ala375	CC CT TT	3 0 34	11 0 28	7 0 30
rs878870695	m.6371 C>T	Synonymous Variant	Ser156	CC CT TT	37 0 0	38 0 1	37 0 0
rs201395766	m.6260 G>A	Synonymous Variant	Glu119	GG GA AA	36 0 1	38 0 1	37 0 0
rs2124593224	m.6068 C>T	Synonymous Variant	Asn55	CC CT TT	36 0 1	39 0 0	37 0 0
rs879118820	m.5999 T>C	Synonymous Variant	Ala32	TT CT CC	37 0 0	37 0 2	37 0 0
rs879104796	m.6047 A>G	Synonymous Variant	Leu48	AA AG GG	37 0 0	37 0 2	37 0 0
rs1556423059	m.5973 G>A	Missense Variant	Ala24Thr	GG GA AA	36 0 1	39 0 0	37 0 0
rs1603220225	m.6018 G>A	Missense Variant	Ala39Thr	GG GA AA	37 0 0	38 0 1	37 0 0
rs201262114	m.6261 G>A	Missense Variant	Ala120Thr	GG GA AA	37 0 0	33 0 6	37 0 0
rs200165736	m.6253 T>C	Missense Variant	Met117Thr	TT TC CC	37 0 0	38 0 1	37 0 0
rs1603220429	m.6340 C>T	Missense Variant	Thr146Ile	CC CT TT	37 0 0	39 0 0	36 0 1

rs879164161	m.6445 C>T	Missense Variant	Thr181Met	CC CT TT	37 0 0	39 0 0	36 0 1
rs1556423267	m.7309 T>C	Missense Variant	Ile469Thr	TT TC CC	37 0 0	39 0 0	36 0 1
rs200784106	m.6663 A>G	Missense Variant	Ile254Val	AA AG GG	36 1 0	39 0 0	37 0 0

Table 11; Genotype frequencies of cytochrome oxidase 2 gene (CO2) variants in non-smokers, tobacco-smokers, and Cannabis-smokers' cases

SNPs ID	Position	Mutation type	Amino acid change	Genotype	NS (N=37)	TS (N=40)	CS (N=38)
rs1603221136	m.7783 T>C	Synonymous Variant	Thr66	TT	37	39	34
				TC	0	0	0
				CC	0	0	3
rs1556423330	m.7705 T>C	Synonymous Variant	Tyr40	TT	36	39	36
				CT	1	0	1
				CC	0	0	0
rs1603221150	m.7810 C>T	Synonymous Variant	Leu75	CC	37	39	36
				CT	0	0	1
				TT	0	0	0
rs386829014	m.7789 G>A	Synonymous Variant	Leu68	GG	36	39	36
				GA	0	0	0
				AA	1	0	1
rs1556423319	m.7657 T>C	Synonymous Variant	His24	TT	37	37	37
				TC	0	0	0
				CC	0	2	0
rs368038563	m.7771 A>G	Synonymous Variant	Glu62	AA	32	38	36
				AG	0	0	0
				GG	5	1	1
rs879161183	m.7873 C>T	Synonymous Variant	Thr96	CC	37	38	37
				CT	0	0	0
				TT	0	1	0
rs1603221066	m.7660 T>C	Synonymous Variant	Asp25	TT	37	38	37
				TC	0	0	0
				CC	0	1	0
rs1556423316	m.7645 T>C	Synonymous Variant	Leu20	TT	37	37	36
				TC	0	0	0
				CC	0	2	1
rs3021089	m.8251 G>A	Synonymous Variant	Gly222	GG	37	36	36
				GA	0	0	0
				AA	0	3	1

rs2068706923	m.8062 C>A	Synonymous Variant	Val159	GG GA AA	37 0 0	39 0 0	36 0 1
rs1603221307	m.8140 C>T	Synonymous Variant	Thr185	GG GA AA	37 0 0	39 0 0	36 0 1
rs879119797	m.7805 G>A	Missense variant	Val74Ile	GG GA AA	37 0 0	38 0 1	36 0 1
rs386420037	m.7853 G>A	Missense variant	Val90Ile	GG GA AA	36 0 1	39 0 0	36 0 1
rs878897170	m.7830 G>A	Missense variant	Arg82His	GG GA AA	37 0 0	39 0 0	36 0 1
rs1556423339	m.7754 G>A	Missense variant	Asp57Asn	GG GA AA	37 0 0	39 0 0	36 0 1
rs1603221063	m.7650 C>T	Missense variant	Thr22Ile	CC CT TT	37 0 0	39 0 0	36 1 0

Table 12; Genotype frequencies of cytochrome oxidase 3 gene (CO3) variants in non-smokers, tobacco-smokers, and Cannabis-smokers' cases

SNPs ID	Position	Mutation type	Amino acid change	Genotype	NS (N=37)	TS (N=39)	CS (N=37)
rs9743	m.9698 T>C	Synonymous Variant	Leu164	TT	34	38	35
				TC	0	0	0
				CC	3	1	2
rs1603222253	m.9335 C>T	Synonymous Variant	Leu43	CC	37	39	36
				CT	0	0	0
				TT	0	0	1
rs374335946	m.9266 G>A	Synonymous Variant	Gly20	GG	37	38	34
				GA	0	0	0
				AA	0	1	3
rs386829084	m.9548 G>A	Synonymous Variant	Gly114	GG	37	39	36
				GA	0	0	0
				AA	0	0	1
rs2248727	m.9540 T>C	Synonymous Variant	Leu112	TT	26	34	31
				TC	0	0	0
				CC	11	5	6
rs1556423706	m.9656 T>C	Synonymous Variant	Ser150	TT	34	38	36
				TC	0	0	0
				CC	3	1	1
rs2853824	m.9347 A>G	Synonymous Variant	Leu47	AA	36	36	37
				AG	0	0	0
				GG	1	3	0
rs2856985	m.9755 G>A	Synonymous Variant	Glu183	GG	36	36	37
				GA	0	0	0
				AA	1	3	0
rs375478739	m.9509 T>C	Synonymous Variant	Phe101	TT	37	38	37
				TC	0	0	0
				CC	0	1	0
rs1603222347	m.9497 T>C	Synonymous Variant	Phe97	TT	37	37	37
				TC	0	0	0

				CC	0	2	0
rs879070193	m.9297 C>T	Synonymous Variant	Leu31	CC CT TT	37 0 0	38 0 1	37 0 0
rs386829074	m.9329 G>A	Synonymous Variant	Thr41	GG GA AA	37 0 0	39 0 0	36 0 1
rs879229894	m.9962 G>A	Synonymous Variant	Leu252	GG GA AA	37 0 0	39 0 0	34 0 3
rs1556423681	m.9495 T>C	Missense Variant	Phe97Leu	TT TC CC	37 0 0	38 0 1	37 0 0
rs1603222339	m.9481 T>C	Missense Variant	Phe92Ser	TT TC CC	37 0 0	38 1 0	37 0 0
rs1556423726	m.9801 G>A	Missense Variant	Val199Met	GG GA AA	37 0 0	38 0 1	36 0 1
rs1556423714	m.9738 G>A	Missense Variant	Ala178Thr	GG GA AA	37 0 0	37 0 2	37 0 0
NOT RECOEDRD/co3	m.9758 T>G	Synonymous Variant	Ser184	TT TG GG	36 0 1	39 0 0	37 0 0
NOT RECORDED/co3	m.9657 C>T	Synonymous Variant	Leu151	CC CT TT	37 0 0	38 0 1	37 0 0
NOT RECORDED/co3	m.9689 A>G	Missense Variant	Leu161 Gln	AA AG GG	37 0 0	38 0 1	37 0 0

4.4 Figures

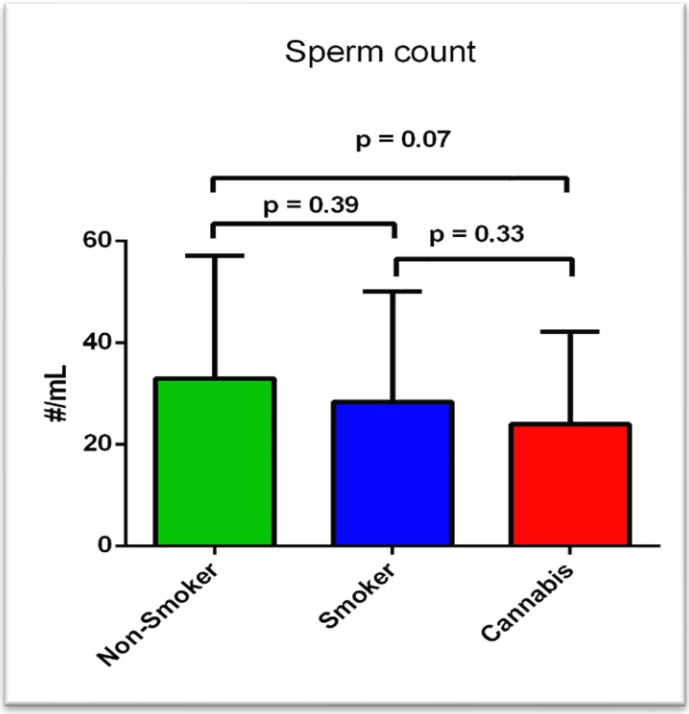


Figure 14; Difference of the sperm count (x106/ml) between the three groups

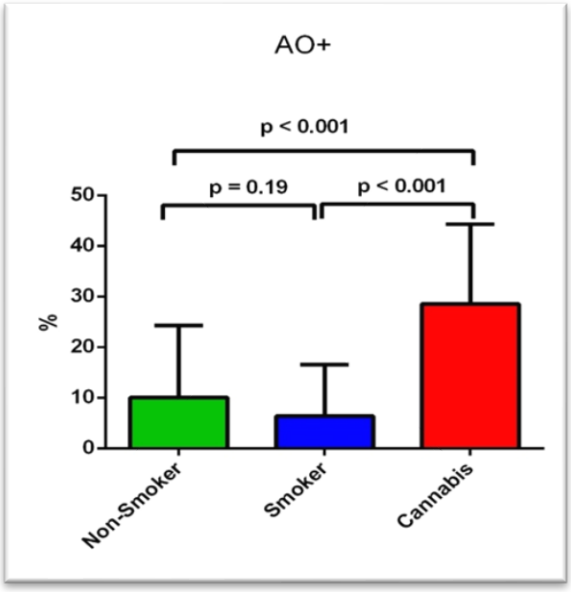


Figure 15; The difference of the proportion of spermatozoa with DNA denaturation assessed by acridine orange test between the three groups

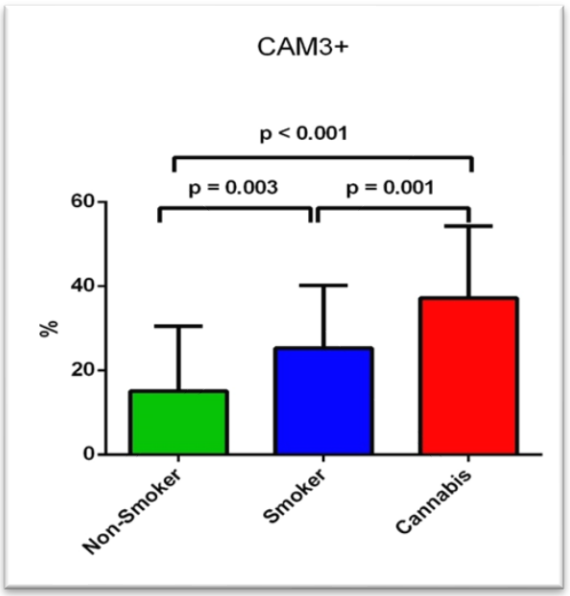


Figure 16; The difference of the proportion of spermatozoa with defective chromatin protamination assessed by Chromomycine CMA3 test between the three groups

Figure 17; Prevalence of MTC-CO1 SNPs in Each group

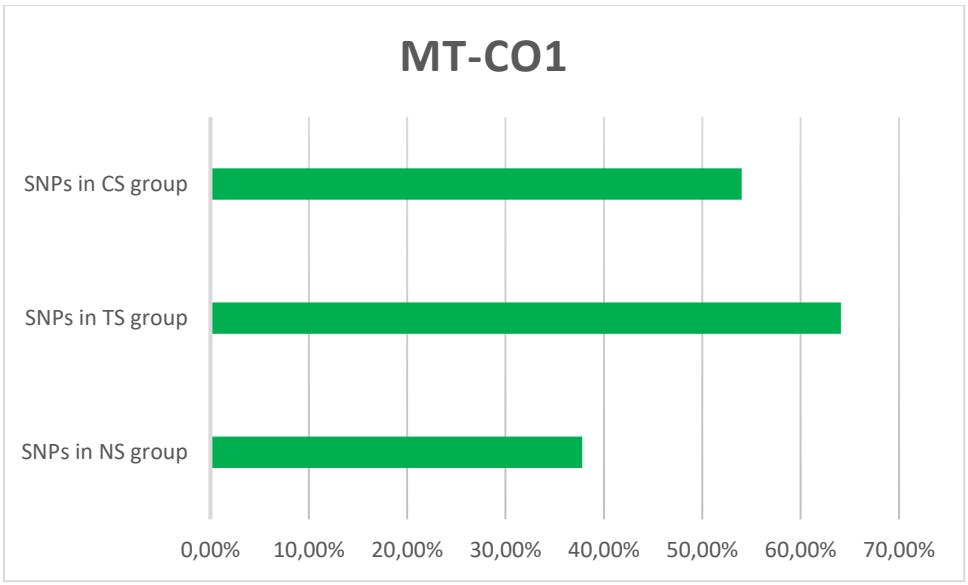


Figure 18; Prevalence of MTC-CO2 SNPs in Each group

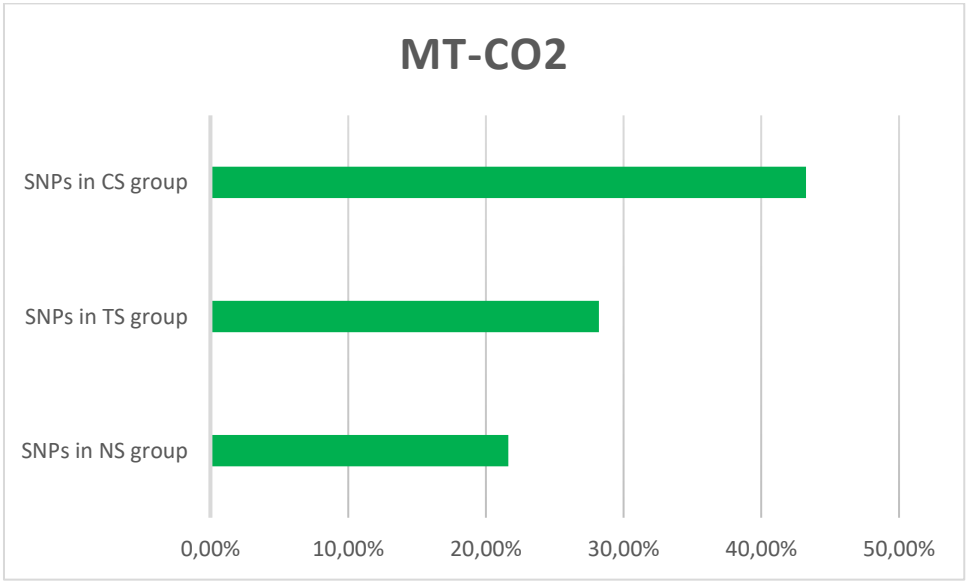
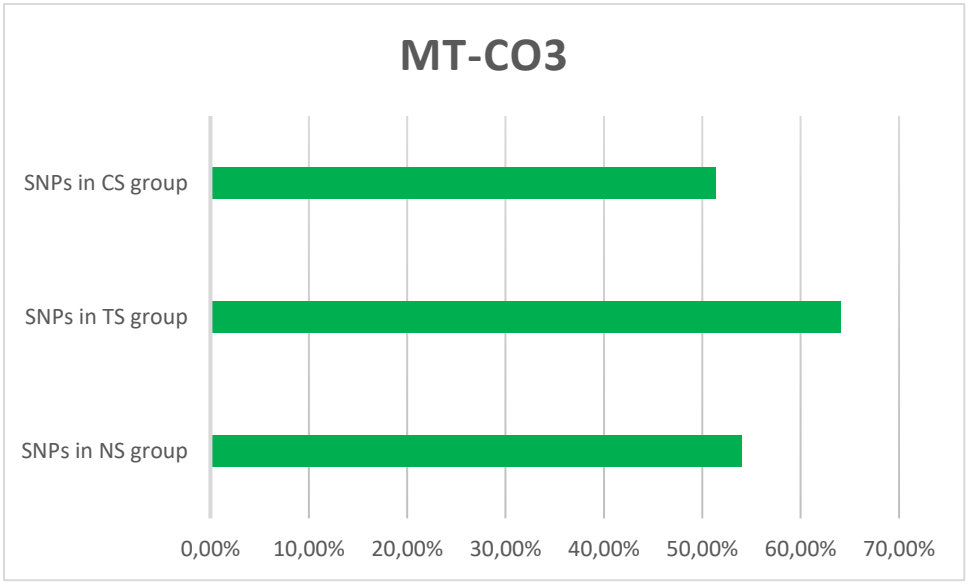


Figure 19; Prevalence of MTC-CO3 SNPs in Each group



5. Discussion:

Tobacco smoking is one of the major factors contributing to male infertility (Daumler et al., 2016), and surveys have indicated that approximately 120,000 young men (ages 30 to 50) in the United Kingdom are impotent because of this harmful habit. Studies on male smokers in infertile couples showed a lower ejaculate volume, despite them having higher testosterone levels (Lotti et al., 2015).

Mounting evidence suggests that tobacco smoking substantially affects both the quality and quantity of sperm, thereby contributing to male infertility (Fuentes et al., 2003; Ricci et al., 2017). Conversely, some studies report no link between smoking and male infertility (Dunphy et al., 1991). Nevertheless, the impact of smoking on sperm motility is still a subject of debate (Bundhun et al., 2019).

Earlier studies showed the adverse effect of cigarette smoking on the progressive sperm motility and sperm morphology irrespective of the total number of cigarettes smoked daily (Colagar et al., 2007; Hassa et al., 2006; Hammadeh et al., 2001; Abdul-Ghani et al., 2014).

Cigarette smoking has been found to detrimentally impact a range of conventional semen parameters, as well as sperm chromatin condensation and viability. Moreover, these negative effects correlate with both the quantity of cigarette smoked daily and the overall duration of smoking. In a study conducted by Yu et al. in (2014), it was observed that the rate of histone abnormalities was significantly lower in non-smoker men with normal sperm counts, while the highest rates were found in heavy smokers with oligospermia within the Chinese population studied (Yu et al., 2014).

Moreover, it has been reported that cigarette smoking was found to also affect Ca2+- ATPase activity of the spermatozoa as well (Kumosani et al., 2008). In addition, different proteins (Aldoa, ATP5a1, Gpx4, Cs) expressed in sperms were significantly altered in smokers (Chen et al., 2015). Based on previous studies, tobacco smoke has a wide range of harmful effects on male reproductive parameters and genome integrity (Al Khaled et al., 2018; Laqqan et al., 2018; Amor et al., 2022).

Furthermore, oxidative stress and its markers such as ROS, malondialdehyde, and 8-hydroxyguanosine (8-OHdG), were significantly higher ($p<0.010$) in smokers than in non-smokers and correlated significantly ($p<0.050$) with P1:P2 ratios (Hammadeh et al., 2010).

The present results are in consistent with other studies conducted by (Cho et al., 2005) who demonstrated that tobacco consumption may contribute to the progression of male infertility

due to alterations in sperm characteristics. A comprehensive investigation compared 1165 male participants aged between 16 and 29 years was carried out in 2003-2004, showed that smoking exhibited detrimental effects on both sperm concentration and total sperm counts; significantly lower sperm concentrations were recorded for smokers (median 60 versus 66 x 10^6/ml, P=0.02) along with reduced total sperm counts (177 versus 226 x 106, P<0.001) in comparison to non-smokers (Cho et al., 2005). Besides, these results are in agreement with others presented by Sharma et al. (2016) who verified the detrimental effects of smoking on sperm parameters and concluded that a substantial reduction in sperm concentration and motility could be observed among smokers compared to non-smokers (Sharma et al., 2016).

Reduced sperm counts, motility, viability, morphology, and inhibition of the acrosome reaction in humans can have drastic implications in the long term with regards to impairing male reproduction as well as negatively impacting the offspring (Bari et al., 2011; Fronczak et al., 2012). Collectively, these findings underscore the significant negative consequences of tobacco smoking exerts on sperm cell function and fertility potential.

Numerous studies have provided insights into the multiple pathways through which tobacco smoking affects sperm cells. One salient mechanism is the direct toxicological impact of the constituents of cigarette smoke on testicular function (Mostafa et al., 2006). For instance, it has been discovered that nicotine impairs spermatogenesis by inducing oxidative stress, DNA damage, and apoptosis within germ cells, leading to reduced sperm count and motility (Jorsaraei et al., 2008; La Vignera et al., 2016;). Additionally, tobacco smoking is known to increase seminal reactive oxygen species (ROS) levels, which disrupts sperm functionality and results in decreased fertilization capacity (Pasqualotto et al., 2008).

Other possible mechanisms might be related to the negative impact of smoking on the 8 nAChR subunits found in human spermatozoa, resulting in smoking-related sperm damage (Condorelli., 2018).

THC is known to bind to cannabinoid receptors in the testes, which can lead to decreased sperm motility, altered sperm morphology, and DNA. These effects may be due in part to the disruption of the endocannabinoid system, which plays a key role in regulating male reproductive function (Schuel et al., 2002).

Although, the common adverse effects of cannabinol (CBD1) are well documented, and they include sleepiness, fever, decreased appetite, ataxia, vomiting, and abnormal behavior and sedation (Devinsky et al., 2018; Szaflarski et al., 2018). Still, little is known about the long-

term effect of (Cannabidiol) on hormone regulation, cognition/memory, fertility, and pregnancy (Sekar and Pack., 2019).

Initially, there was lack of clarity regarding the presence of CB2 receptors in spermatozoa, but a study conducted by Maccarrone et al. (2003) confirmed the presence of the CB2 receptor in the postacrosomal region, midpiece, and tail of human spermatozoa. CB2 receptors were also found to be present on Sertoli cells (Maccarrone et a., 2003).

Previous study revealed that daily administration of 12.5 mg/kg of cannabis for 30 days led to complete spermatogenesis arrest (Eisenberg et al., 2014).

There is a growing body of evidence suggests that cannabis use may have adverse implications for male reproductive health, particularly regarding the viability and functionality of sperm. Gundersen documented a significant decrease in sperm concentration among cannabis users compared to non-users (Gundersen et al., 2015), while another study by Huang et al. (1978) observed altered sperm morphology and DNA integrity from cannabis exposure (Huang et al., 1978).

It was also observed that the concentration and motility of sperm were negatively affected by frequent cannabis usage (Wenger T et al., 1987). In an extensive literature review (Cho et al., 2005) revealed a clear association between chronic cannabis use and reduced sperm count, sperm motility, and altered sperm morphology. The impact of cannabis on the motility of spermatozoa has been observed to decrease at both therapeutic and recreational levels of tetrahydrocannabinol (THC) (Whan et al., 2006).

Du Plessis et al., (2015) investigated the underlying mechanisms and revealed that the primary psychoactive compound in cannabis, Δ-9-tetrahydrocannabinol (THC), could disrupt key signaling pathways involved in spermatogenesis and impair testosterone production (Du Plessis et al., 2015). Consequently, this impairment may lead to reduced spermatogenic capacity and suboptimal sperm function. The accumulating research underscores the importance of understanding the potential risks associated with cannabis smoking on male fertility and highlights the need for further investigations to elucidate the full extent of its impact on sperm cells (Nassan et al., 2019).

It should be noted, however, that conflicting results exist in the literature regarding THC's influence on sperm quality. Notably, research conducted by Gundersen et al. in 2015 observed no significant variances in terms of sperm count, motility, or structure when comparing marijuana consumers with individuals who do not use the substance (Gundersen et al., 2015).

These discrepancies may be due to differences in study design, sample size, or participant characteristics (Klonoff-Cohen et al., 2006).

The finding of this study confirms with previous study conducted on human by Hembree et al., (1980) and other experimental study on animals (Banerjee et al., 2011) who demonstrated that sperm concentration decreased after exposure to cannabis. It also appears that sperm counts are inversely proportional to the amount of drug taken (Kolodny et al., 1974). Moreover, a large-scale prospective study by (Nassan et al., 2019) demonstrated a notable link between regular cannabis smoking and lower total sperm count in men when compared with individuals who had never smoked cannabis.

Pacey et al, conducted a prospective trail included, 1,700 unmatched case reference study of men in the United Kingdom presenting at a total of 14 fertility clinics and found that men who had used cannabis in the 3 months prior to collection of a semen sample were more likely to have poor sperm morphology, defined as less than 4% morphologically normal spermatozoa (OR 1.94, 95% CI 1.05e-3.60) (Pacey et al., 2014).

Moreover, the findings of the present study are consistent with previous research that has demonstrated the negative effects of tetrahydrocannabinol (THC) on human sperm function and DNA integrity (Whan et al., 2006; Barbonetti et al., 2010).

It is crucial to mention that cannabis smoke has been shown to have numerous toxicants and carcinogens including acrylonitrile (a potential carcinogen carbon monoxide cardiovascular toxin),6 and 1,3, -butadiene (a carcinogen) (Wei et al., 2016). Very few published studies have examined cannabis smoke metabolites in humans (Wei et al., 2018).

The present data showed current or past Cannabis users had more damaged sperm, lower sperm counts and reduced the mean morphologically normal spermatozoa in comparison to cigarette smoking.

Previous study (Hembree., 1980) showed a similar decrease in sperm motility in an analysis of semen samples from 16 healthy, chronic marijuana users after 4 weeks of high dose marijuana. Barbonetti et al elucidated the mechanism of these findings by establishing a link between CB1 and sperm mitochondrial activity (Barbonetti et al., 2010).

A follow-up study examined the effects of heaviness of cannabis smoking (ie, joints per day/month/year and never use) on polycyclic aromatic hydrocarbons (PAH) levels. Results indicated cannabis smokers who smoked two or more joints per day demonstrated higher levels of 2-hydroxynaphthalene, 2-hydroxyfluorene, and 1-hydroxypyrene than less frequent cannabis

smokers (Wei et al., 2016). These studies indicate that individuals who smoke cannabis are subject to exposure to harmful toxic substances. As a result, those who concurrently use cannabis and tobacco could potentially face a greater risk of exposure to toxicants and carcinogens compared to those who only smoke cigarettes.

It is worth noting that the effects of marijuana on male reproductive function may be reversible with cessation of use. A study in 2018, found that men who stopped using marijuana for at least 74 days had significantly higher sperm concentration and motility compared to those who continued to use marijuana (Qureshi et al., 2018).

Since the COVID-19 pandemic, the prevalence of substance use, including tobacco products, marijuana, opioids, and alcohol, has been increasing, especially among men of reproductive age (Villanueva-Blasco et al., 2024). In 2021, 44% of adults surveyed believed smoking marijuana every day is safer than smoking tobacco every day, compared to about 37% in 2017 (Chambers J et al., 2023).

It is widely accepted that unhealthy lifestyles, including drinking alcohol and using tobacco or marijuana products, can negatively affect overall health. However, his trend of increased consumption is concerning because men are often unaware of the negative impact this may have on their reproductive health. Available evidence suggests that substance use may affect spermatogenesis, secretion of reproductive hormones through the hypothalamic-pituitary-gonadal (HPG) axis, and sexual function (Pollock et al., 2021; Wang et al., 2022; Amor, 2022, 2021).

To our best knowledge, this is the first study that aims to compare and to determine the effects of tobacco and cannabis smoking in association with variants in the Cytochrome C Oxidase 1, 2 and 3 genes (MT-CO1, MT-CO2, and MT-CO3), on spermatozoa function and male fertility.

The findings of the present study showed that normal sperm morphology was significantly lower in tobacco smokers and cannabis smokers in comparison to non-smokers ($p<0.001$). Moreover, there was a significant reduction in normal sperm morphology in the cannabis smoker group compared to the tobacco smoker group ($p = 0.002$).

Sperm progressive motility showed a non-significant reduction in the cannabis-smoking group compared to tobacco smoking and the non-smoking group ($14.27 \pm 11.3\%$) ($p=0.223$). Moreover, non-progressive motility showed a significant reduction in the cannabis-smoking group and the tobacco-smoking group compared to the non-smoking group ($p < 0.001$).

However, the mean percentage of immotile sperm was significantly higher in the cannabis-smoking group compared to tobacco smoking and the non-smoking group ($p < 0.001$).

On the other hand, sperm count, and semen volume showed no significant difference between the three groups (p=0.199; p=0.091, respectively).

We investigated the sperm DNA integrity using acridine Orange (AO) assay and chromomycine staining (CMA_3). The results showed a significant increase in the AO+ score in the cannabis-smoking group (28.53 ± 15.8%) compared to the non-smoking group (10.1 ± 14.2%) and the tobacco-smoking group (6.4 ±10.2%; $p < 0.001$). At the same time, there was a significant difference in AO+ score between the tobacco-smoking and non-smoking groups ($p = 0.001$).

In addition, the CMA_3+ test showed a significant increase in the CMA_3+ score in the cannabis-smoking group compared to the non-smoking group ($p < 0.001$) and between the tobacco-smoking group compared to the non-smoking group ($p = 0.003$). At the same time, there was a significant increase in CMA3+ score cannabis-smoking men in comparison to tobacco smoking men ($p = 0.001$).

These results are in accordance with a previous study showing that smoking negatively alters the sperm standard parameters, DNA stability of sperm and the ratio of protamine mRNA as well as downregulates the expression of H2BFWT, PRM1 and PRM2 (Amor et al., 2021). Moreover, several studies have reported that the spermatozoa of tobacco smokers have higher levels of DNA fragmentation in comparison with non-smokers (Amor et al., 2021 Gene expression). Numerous studies have provided insights into the multiple pathways through which tobacco smoking affects sperm cells. For instance, it has been discovered that nicotine impairs spermatogenesis by inducing oxidative stress, DNA damage, and apoptosis within germ cells, leading to reduced sperm count and motility (Jorsaraei et al., 2008; La Vignera et al., 2016).

Also, Cigarette smoking has been found to detrimentally impact sperm chromatin condensation and viability. Moreover, these negative effects correlate with both the quantity of cigarette smoked daily and the overall duration of smoking. In a study conducted by Yu et al, it was observed that the rate of histone abnormalities was significantly lower in non-smoking men with normal sperm counts, while the highest rates were found in heavy smokers with oligospermia within the Chinese population studied (Yu et al., 2014). In addition, excessive ROS production can lead to oxidative stress, which in turn affects sperm nuclear DNA, sperm mitochondrial respiratory activity (Piomboni et al., 2012) and endocrine function, leading to a variety of male reproductive system diseases. This may lead to male infertility (Darbandi et al., 2018).

The effects of smoking and passive smoking on various sperm parameters have been studied (Hammadeh et al., 2010; Sharma et al., 2016; Bundhun et al., 2019). Decreases in sperm density, motility, and possible negative effects on morphology, have been demonstrated (Harlev et al., 2015). Sperm concentration decreased by an average of 22% in a dose-dependent manner. Smokeless tobacco consumption also has a dose-dependent negative effect on some sperm parameters (Said et al., 2005). Although sperm concentration, motility, and/or morphology are reduced compared to nonsmokers, they generally remain within normal ranges. However, available evidence suggests that smoking may negatively affect sperm binding to the zona pellucida. These findings are based on a study using a penetration test with zona-free hamster eggs (Sofikitis et al., 2000).

Consistent with our findings, sperm morphology and motility are the parameters most frequently examined in studies about marijuana use of men attending fertility centers. In three studies conducted in Jamaica, the Pacific Northwest, marijuana use (past, past 3 months, and current) was associated with an increased risk of abnormal sperm morphology (Pacey et al., 2014; Carroll et al., 2017). Cannabis compounds can have a significant impact on sperm motility in male participants. As our in-vitro study results suggest that the high dose of Cannabis can lead to a decrease in sperm motility, which may have implications for male fertility.

In contrast, in a study of current, past ever, and never marijuana men users were, no significant association with percentage of normal morphology was found. The risk of morphological abnormalities was also lower compared with men who had never smoked marijuana.

Three studies on sperm motility also had conflicting results. Although heavy or recent marijuana use was associated with an increased risk of “abnormal motility” in Jamaican men (Carroll et al., 2017), no significant associations were found between different categories of marijuana use and total sperm motility percentage (Nassan et al., 2019).

There is a growing body of evidence suggests that cannabis use may have adverse implications for male reproductive health, particularly regarding the viability and functionality of sperm. Gundersen et al, documented a significant decrease in sperm concentration among cannabis users compared to non-users (Gundersen et al., 2015), while another study by Rajpert-De Meyts et al, observed altered sperm morphology and DNA integrity from cannabis exposure (Rajpert-De Meyts et al., 2016).

Moreover, we aimed in the present study to investigate the potential genetic alterations within the genes that encodes for the mitochondrial cytochrome c oxidase (Complex IV) (MT-CO1, MT-CO2, and MT-CO3) in the sperm cells of individuals who consume tobacco and cannabis.

This gene is crucial for cellular respiration and energy production within the cell, and any variations could significantly impact cellular performance. Given the widespread use of tobacco and cannabis, understanding their potential impact on genetic integrity and fertility is important. A total of 22 SNPs in MT-CO1 were identified; 14 of them were synonymous variants, while eight were missense mutations.

The percentage of men with total variants in the CO1 gene groups NS, TS, and CS were: 37,8 %, 64,1 %, and 54 % respectively. However, none of these SNPs were significantly different between these groups (p=0.10). In addition, 17 genetic alterations in MT-CO2 gene were identified; Twelve of them were synonymous variants and five of them were missense mutations.

The percentage of men with total variants in the CO2 gene groups NS, TS, and CS were: 21,62 %, 28,2 %, and 43,24 % respectively. None of these SNPs were significantly different between these groups (p=0.23).

Besides, 20 genetic variations in MT-CO3 were identified; 13 of them were synonymous variants and four of them were missense mutations and Three SNPs were not listed in the NCBI database.

The percentage of men with total variants in the CO3 gene groups NS, TS, and CS were: 54,05 %, 64,1 %, and 51,35 % respectively. Also, none of these SNPs were significantly different between these groups (p=0.07).

The results of the present study however, are in accordance and confirm our previous study showing that smoking had no linkage with genetic variants in the H2B histone family member W, testis-specific gene: H2BFWT, and protamine PRM1 and PRM2genes (Amor et al., 2021). Nevertheless, Saleh J et al., (2022) suggested that male subfertility is linked with rs7520428 SNP in MT-CO3 (Saleh J et al., 2022).

Tobacco smokers undergo a substantial build-up of genetic mutations due to the complex chemical composition of tobacco smoke (US Department of Health and Human Services, 2020). In another study, Dasgupta et al., (2012) observed significantly higher mtDNA mutation in the never-smokers compared to the current-smoker lung cancer patients (P = 0.006). mtDNA mutation was significantly higher in the never-smoker Asian compared to the current-smoker Caucasian patients' population (p = 0.026). They also observed a significant increase in mtDNA content among the never-smoker lung cancer patients (P = 0.037) (Santanu Dasgupta et al., 2012).

The results of our in vitro study showed a significant reduction in sperm motility following exposure to both THC and CBD was observed.

These findings are in accordance with a previous study conducted by Murphy et al who investigated the impact of *Cannabis* compounds on male reproductive health and showed that an acute exposure to THC deteriorate sperm function and consequently fertilization potential (Murphy et al., 2018).
Nonetheless, previous study demonstrated that chronic exposure to low concentrations of THC imitated an endocannabinoid-related reduction in mouse sperm motility and viability (López-Cardona et al., 2018).

Furthermore, the present research elucidated that exposure to CBD resulted in diminished sperm motility, even though it was to a lesser extent compared to the influence of THC.
Nevertheless, numerous studies have confirmed alterations in endocannabinoid signaling within male reproductive tissues, indicating that signaling modulation of male reproductive system by exogenous cannabinoids could have substantial consequences of sperm function (Franzoni et al., 2020).
This study examined the consequences of THC-COOH, THC, and CBD on male sperm (n=10) with participants aged twenty to thirty. Results revealed that both fractions exhibited diminished motile sperm quantities; however, THC and CBD inflicted more significant damage than sperm in the THC-COOH fraction.

The endocannabinoid system and its endogenous compounds exhibit diverse physiological functions at both cellular and organ levels (Huestis., 2007). A functional endocannabinoid system is present in various segments of the human reproductive tract, such as endometrium, ovary, epididymis, testis, sperm, and prostate. Identified endogenous agonists include anandamide (AEA), oleoylethanolamide, and palmitoylethanolamide, which are detected in reproductive secretions (Whan et al., 2006). Cannabinoid receptors have been demonstrated to influence menstrual cycles, implantation, embryonic development, lactation, and pregnancy maintenance in females (Ghosh and Rai 2018). In contrast, information regarding the role of cannabinoid receptors in male fertility remains inadequate. However, recent studies provide indirect evidence for the existence of cannabinoid pathways in male sperm analogous to those found in the central nervous system by demonstrating the binding of labeled agonists (Payne et al., 2019 and Rajanahally et al., 2019).

In Vitro, examinations have investigated the consequences of THC on human sperm functionality. This specific cannabinoid exerts a dose-responsive adverse impact on progressive motility percentage, becoming more highlighted as semen quality diminishes overall. Furthermore, spontaneous acrosome reactions experience reduction, and THC demonstrates the capacity to impede the acrosome reaction even when artificially induced at both therapeutic and recreational plasma concentrations (Rajanahally et al., 2019).

Contradictory findings pertaining to decreased testosterone levels, sperm production, sperm motility, and elevated sperm abnormalities (Kolodny et al., 1976) were later challenged by a more extensive and methodically rigorous study encompassing chronic-heavy users. This investigation revealed no discernable differences in plasma testosterone either at the beginning of the study or following three weeks of substantial daily *Cannabis* consumption (Mendelson et al., 1974). In the literature scanty information in this regard could be found. The potential anti-motility effects of CBD provide an intriguing area for further investigation within this field, warranting additional research and potential interactions with THC and other cannabinoids.

This study provides compelling evidence and supports the findings on how THC negatively affects sperm mobility.

6. Conclusions

The current research demonstrated that both cigarette and cannabis consuming causes a deleterious effect on sperm concentration, motility, vitality, maturation, DNA fragmentation, and DNA-RNA Oxidative damage. However, more deterioration of spermatozoa quality was shown by comparing cannabis smokers to cigarette smokers and non –smokers. Also, cannabis smoking is more harmful for spermatozoa than tobacco smoking. Besides, the lack of a correlation between the identified variant alleles and each of non-smoker, tobacco smokers and cannabis smoker classes demonstrate that smoking seems unlikely to alter the nucleotide sequence of these genes rather than sperm DNA.

Further research is needed to confirm these findings and determine the extent to which Cannabis use may impact male fertility. Nonetheless, these results highlight the potential risks associated with Cannabis use and underscore the need for caution when using this substance.

While cannabis may not be as toxic or harmful as many other substances, whether herbal, synthesized, or medicinal, it remains, for sure, the most widely used drug worldwide and is particularly easy to develop a chronic habit with. This study examined how cannabis negatively impacts cellular vitality, with specific observations on sperm cells. Although its effects do not necessarily lead to impotence or total infertility in men, the potential implications are significant enough to warrant comprehensive investigation, especially given its widespread use. It is crucial to explore the long-term effects of cannabis use, as many individuals consume it daily over e xtended periods. Understanding its influence on our genetic makeup is crucial in contemporary society, particularly as cannabis becomes increasingly legalized and used medicinally. Especially, when it comes to our knowledge that, long-term or frequent cannabis use has been associated with an increased risk of psychosis or schizophrenia in some individuals (Volkow ND et al., 2016).

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