INVESTIGATING THE IMPACTS OF EXOGENOUS OPIOIDS ON MALE REPRODUCTIVE HEALTH

by

IAN DAVID BACHLI

(Under the Direction of Charles A. Easley IV)

ABSTRACT

In 2021, approximately 60 million people used opioids worldwide. Among them, nearly 40 million live with drug use disorders. Opioid use and overdose have increased in recent years across several countries alongside its devastating impact on the United States. This opioid epidemic was initially attributed to the overabundance and overprescription of opioids for use in the management of chronic pain but has now shifted towards the abuse of highly potent opioids such as fentanyl appearing on the illicit drug market. Given the prevalence of male opioid users combined with both the addictive properties and the known detrimental health effects of these substances, there is a need to understand the potential consequences of opioid exposure on male reproductive health. Notably, global fertility rates have declined, and both semen parameters and sperm counts have been on a decline. Studies have highlighted the importance of environmental exposures as well as lifestyle choices in determining health outcomes over time. Emerging evidence suggests that exogenous opioid use in men reduce semen quality and induce DNA damage, hypogonadism, and DNA hypermethylation. While endogenous opioids are suggested to play a role in male reproductive function, there is little known on the direct effects these exogenous opioids on both the blood-testis barrier, a critical feature of this tightly regulated system, as well as the germ cells and their epigenome during development. To assess the effects of exogenous opioids on male reproductive health, we exposed critical male reproductive cells to pharmacologically relevant doses of Fentanyl using a novel *in vitro* human blood-testis barrier model alongside an *in vitro* human spermatogenesis model to investigate the consequences originating from opioid use.

INDEX WORDS: Opioids, Opioid Epidemic, Fentanyl, Male Reproductive Health, Infertility, Epigenome, Blood-Testis Barrier, Spermatogenesis

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DEDICATION

This dissertation is dedicated to those who strive to make the world a better place for all the living things within it. While there are a multitude of complex, challenging issues in the world we live in, changes, no matter how small, can make an impact. After all, big things have small beginnings.

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CHAPTER 1

DESCRIPTION OF DISSERTATION STRUCTURE

Serving to fulfill the requirements set in the University of Georgia's Theses and Dissertations Student Guide to Preparation and Processing (revised in November 2021) for using regular and journal articles as chapters, this introductory chapter serves to inform the reader of the structure of this dissertation. The chapters included in this dissertation that follow the normal structure are Chapters 1 – 5 to maximize readability and cohesiveness across the board.

Chapter 2, "Introduction (Literature Review)" was derived from a co-author review article "Influence of substance use on male reproductive health and offspring outcomes" published in Nature Reviews Urology[1]. While changes were made to prepare my work for that article in order to meet the journal's guidelines, here, that work has been greatly expanded upon to encompass the focus of this dissertation. While at the time of writing this chapter (April 2025), the article is not yet free, however, a copy of the work has been included in Appendix B for reference in the official formatting style of the journal.

Chapters 3, 4, and 5 are written as normal chapters within the guidelines of the dissertation format. However, chapters 3 "The Impacts of Opioids on the Blood-Testis Barrier" and 4 "The Impacts of Opioids on Spermatogenesis and the Sperm Epigenome" will be modified into manuscripts and prepared for submission to publication at a later date. Changes are expected to be made during this preparation process including removal of unnecessary information, changing the title, and revising the figures for succinctness, however, those chapters both remain representative of the work that will be used for submission and may be expanded upon for future studies. Chapter 5 “Concluding Remarks” serves as a summary of the findings present in this dissertation as well as current state of the opioid crisis and what future work may be required to address the ongoing current issues.

CHAPTER 2

INTRODUCTION (LITERATURE REVIEW)

2.1 CONCERNING REPRODUCTIVE HEALTH

Considering the human population on Earth, an estimated 8.2 billion, it may come as a surprise that fertility rates have been on the decline over the past few decades. Amidst it all, studies have uncovered alarming findings on infertility, highlighting observations on a global scale as well as alongside couples discovering for themselves that they may not be able to conceive children. Globally, nearly 20% of all couples struggle with infertility[2]. In the United States alone, almost 12% of couples have impaired fertility, with over 50% of that infertility being attributed to a male factor[2-4]. While there are a multitude of known factors that have the potential to harm human reproductive health, around 30% of these origins remain idiopathic[5]. In the early 1990's, researchers voiced their concerns on the impact the environment may have on human reproductive health, namely fertility, and have reported an overall decline in both semen parameters as well as sperm counts in the past decade[6]. Investigating further into this phenomenon, researchers looked at Western countries, including Australia, North America, and Europe, and observed this alarming trend. The meta-analysis revealed an almost 60% decline in semen parameters, including sperm counts, in men living in Western countries between the years 1973 and 2013[7]. A recent follow-up study showed this same trend in declining sperm counts was also present among men living in Asia, Africa, as well as South America[8]. The causation behind this accelerating trend in the decline of male reproductive health remains unknown and warrants investigation, and although there is clever work currently ongoing to determine this shift in reproductive health and what factors are to blame for it, it remains a massive concern worldwide.

While there are many known contributors to infertility, namely genetic mutations[9], scientific evidence suggests that the environment can play a massive role in determining reproductive success. To better understand this connection between environment, genetic disposition, and health, researchers have examined associations between various somatic disorders and male reproductive health[10-12], discovering that environmental factors are widespread and can lead to a multitude of health concerns. These environmental factors originate from a wide range of substances, such as exposure to flame retardants and medical interventions[13-18]. Even lifestyle factors such as diet, stress, alcohol, cigarettes, and pharmaceuticals, have been implicated in negative health outcomes [1,19,20]. Alarmingly, many of these environmental insults may not arise until later in life, long after exposure, and thus evaded detection and thus investigation in prior scientific and clinical studies.

To encapsulate this dynamic exposure paradigm and its weight on human health, scientists have proposed a theory known as the developmental origins of health and disease (DOHaD). This concept breaks away from the classical understanding that genetics defined destiny, as Lacagnina puts it, and suggests the origins of many lifestyle-related diseases, like cardiovascular disease, obesity, and diabetes, are established during the prenatal and early childhood stages of life[21-24]. These origins are influenced by the complex interplay between genes and the environment, and moreover, have the potential to modulate genetic function via epigenetic mechanisms and thus modulators in determining human health throughout a lifetime[25]. Recently, this concept has been expanded upon and suggests external factors can not only modify an individual's health, but may also lead to inheritable changes in offspring from maternal and/or paternal origins, all through modulating our epigenetics[26-28].

Regarding this relatively new concept postulating the long-term association between exposures and human health, early-life developmental exposures cannot be overlooked when addressing the idiopathic origins of poor reproduction and infertility. As new evidence continues to emerge, and sperm counts continue to decline, it remains critical to discover and learn how our lifestyles and environment influence our health and wellbeing, and what we can do to both alleviate and prevent damage from these exposures.

2.2 THE OPIOID CRISIS

For nearly three decades, the ongoing opioid crisis has represented one of the most pressing public health challenges in modern times. Initially fueled by the overprescription of opioid painkillers in the late 1990s and ignited by the proliferation of illicit opioids such as heroin and fentanyl, this multifaceted catastrophe has reached epidemic proportions; transcending geographic, socioeconomic, and demographic barriers, claiming thousands of lives each year, leaving a devastating toll on individuals, families, and communities alike. What began as a well-intentioned medical effort to alleviate what medical professionals deemed as the fifth vital sign, pain and suffering; a condition that affects more Americans than diabetes, heart disease, and cancer combined[29], quickly spiraled into a complex and multifaceted problem with far-reaching consequences.

Worldwide, about 60 million people between the ages of 15–64 years used opioids at least once in 2012[30]. From 1999 to 2014 prescription opioid sales in the U.S. nearly quadrupled[31,32]. In 2012, providers wrote 259 million opioid prescriptions, enough for every American citizen to have their own bottle of pills[33,34]. This surge, stemming from a combination of aggressive pharmaceutical marketing and a shift in clinical practice regarding opioids as effective pain management drugs for a wide array of pain-related illness and disease, incidentally, brought a host of problems far outweighing their medical efficacy. Opioids are highly addictive, and have profound effects on the body, especially with chronic use, and can lead to numerous health issues and even death[1,35-39]. In what has been deemed the first wave of the opioid crisis (1990s), addiction and overdose rates climbed, and the public began to realize just how dangerous these medications were. Nationally, death rates from prescription opioid overdoses quadrupled from 1999 – 2000[40]. In response, there was pushback from communities on medical professionals and pharmaceutical companies as this issue was brought to the attention of the public. This change is attributed to the broader awareness of the crisis alongside changes in prescribing practices, prompted by government intervention, evolving medical guidelines, and even legal action against pharmaceutical manufacturers[41-43]. Yet, even at their lowest levels, opioid prescription rates in the U.S. remain high. Recent reports have examined that the overall national opioid dispensing rate declined steadily from a rate of 46.8 opioid prescriptions dispensed per 100 persons in 2019 to a rate of 37.5 opioid prescriptions dispensed per 100 persons in 2023[44,45].

Interestingly, when prescription opiates first began to decline, both addiction rates and overdoses remained high, thus sparking what is known as the second wave of the crisis (2010). With a reduced number of prescription opioids available, the use of illicit drugs, particularly heroin, a more potent opioid, had drastically increased, and alongside it came an increase in the number of fatal overdoses involving heroin[40]. Only a few years later came the third wave of the opioid crisis (2013) along with substantial increases in overdose deaths involving synthetic opioids, particularly those involving illegally manufactured fentanyl and its analogs; opioids far stronger than heroin and morphine. As years passed, overdoses rates continued to climb, and more illicit fentanyl began to surface in the United States. From 1999-2022, nearly 727,000 people died from an opioid overdose[46]. Local, state, and federal governments attempted to mitigate the damage done by pushing through funding, medical clinics, and awareness programs[47], but the crisis had left its scar and threatens to remain a concern despite these remediation efforts.

Even after the staggering death toll and public outcry that spanned the last two decades, opioid abuse and overdose remains a massive problem. In 2023, approximately 125 million opioid prescriptions were dispensed to American patients, and while the number of prescriptions has decreased since the dawn of this epidemic, and now synthetic opioids, such as fentanyl, have become the lead contributors to dramatic increases in abuse and overdose deaths in the United States[48]. Recently, the Centers for Disease Control and Prevention (CDC) reported that drug overdose remains the leading cause of injury mortality. Notably, the mortality rate involving a drug overdose in 2021 was over six times the number of that in 1999; considered the start of the epidemic. Moreover, the number of drug overdose deaths increased more than 16% from 2020 to 2021 alone, with over 75% of the nearly 107,000 drug overdose deaths in 2021 involving an opioid[48]. The Joint Economic Committee estimates the opioid epidemic cost the United States nearly $1.5 trillion dollars in 2020 alone, also noting a substantial increase of 37% from 2017, when the cost had been last measured[49]. This is largely due to the abundance of fentanyl emerging in the United States, largely originating from illicit manufacturing and entering the illegal drug market. In 2023, an estimated 115 million pills containing illicit fentanyl were seized by law enforcement, compared to just under 50 thousand in 2017[50], indicating an evolution of the ongoing opioid crisis and the shift away from prescription opioids towards more powerful synthetic opioids arising from illicit origins.

Although the demographics of the people who use or have used opioids is relatively diverse within the United States[51], opioid-related deaths disproportionately impact younger adults, and particularly men. The CDC noted in a 2023 surveillance report that drug overdose death rates were highest among males aged 35–44, followed closely by those aged 25–34[51]. Another study found that younger male populations and communities with lower socioeconomic status have increased as the main victims of the opioid crisis after synthetic opioids had overtaken both heroin and prescription opioids as the dominant contributors to overdose mortality[52]. Generally, men show higher rates of substance abuse across many age groups among many of the substance categories, including alcohol, tobacco, marijuana, and other illicit drugs [53], highlighting a large percentage of the population at risk not only to opioid abuse and overdose, but the other harmful effects this substance provides. Moreover, this large percentage of the population falls within the reproductive age range, thus warranting question as to what issues may arise in terms of reproductive health, fertility, as well as in offspring.

While the United States has been greatly impacted by the opioid crisis, it has not been the only country that has suffered. Particularly alongside the emergence of illicit fentanyl, other countries have recently noticed similar trends to the United States' opioid epidemic. Canada and several Central American countries have observed increased opioid abuse and overdose closely following that of the United States, likely due to being geographically relative. However, now even numerous European countries as well as Australia are experiencing their own opioid crises[54-56]. Given the now international widespread use of opioids, both prescription and illicit, there is a pressing need to investigate how these potent drugs impact human health after exposure. While mortality remains a great concern, research considering the other consequences is highly warranted, and essential not only for addressing individual health outcomes but also for putting an end to this ongoing threat.

2.3 THE EFFECTS OF OPIOID USE

Within the human body, the endogenous opioid system is a crucial neuroregulatory network responsible for modulating pain perception, stress response, mood, reward, and addictive behaviors. This system functions through the action of endogenous opioid peptides—including endorphins, enkephalins, and dynorphins—that bind to mu (μ), delta (δ), and kappa (κ) opioid receptors found primarily throughout the central and peripheral nervous systems. These endogenous peptides help mediate a variety of physiological and behavioral responses by modulating neurotransmission and dampening pain signals[57,58]. Their release is typically short-lived and situational, such as in response to injury, exercise, or emotional stress, contributing to a balanced physiological response, but may play additional roles throughout various organs and tissues. Importantly, exogenous opioids, such as morphine, oxycodone, heroin, or fentanyl, can mimic endogenous opioids and interact with the endogenous opioid receptors. However, these exogenous opioids often bind with greater affinity and increased durations. For example, fentanyl, a synthetic opioid, is estimated to be 50 to 100 times more potent than morphine and nearly 50 times stronger than heroin, making even small doses potentially lethal[59]. Exposure to these powerful substances results in not only clinically relevant analgesic effects, but also excessive dopamine release in the mesolimbic pathway, especially the nucleus accumbens, producing a euphoric high and reinforcing drug-taking behavior[60]. While exogenous opioids consist of a broad class of drugs; varying by their target receptors, pharmacokinetics, binding affinities, chemical structures, and half-lives, they are typically far stronger and thus have more profound impacts on our bodies compared to the naturally produced opioids within the endogenous opioid system.

Over time, repeated exposure to these exogenous opioids leads to neuroadaptation; a process involving the downregulation of opioid receptors and decreased production of endogenous opioids, and in turn diminishes the natural capacity of the brain to regulate pain and pleasure[61]. This neuroadaptation contributes to tolerance, dependence, and addiction, wherein users compulsively seek opioids despite harmful consequences[62,63]. While this interaction provides potent analgesia, long-term use has been shown to induce adverse consequences across multiple organ systems that have been implicated in health deficits and fatalities.

One of the most immediate and life-threatening consequences of opioid use is respiratory depression. Opioids suppress the brainstem's respiratory centers, diminishing the body's ability to respond to elevated carbon dioxide levels. This can result in hypoventilation, hypoxia, and, in severe cases, fatal overdose[39,64,65]. Respiratory depression is particularly pronounced with high doses, concurrent use of other central nervous system depressants, and when potent opioids such as fentanyl are used. While respiratory depression is the most well-known effect due to its connection to mortality rates in opioid abuse, there are other notable harmful effects brought on through opioid use. For instance, chronic opioid use has also been shown to induce significant gastrointestinal dysfunction, most notably opioid-induced constipation (OIC)[65]. This results from the binding of opioids to receptors in the enteric nervous system, leading to decreased peristalsis and delayed gastric emptying[65,66]. Opioids can also induce nausea and vomiting by stimulating the chemoreceptor trigger zone in the medulla, adding further complications in long-term use[67]. Both opioid-mediated effects have the potential to become severe and may require discontinuation of treatment if within a clinical setting, or additional pharmacologic intervention. However, due to the rate of illicit opioid abuse outside of medical guidance and administration, this complication often remains unaddressed.

The hallmark characteristic of exogenous opioid use is the profound neurological and cognitive effects on the human nervous system, particularly over repeated use. Chronic usage is associated with alterations in brain structure and function, particularly in regions involved in decision-making, memory, and emotional regulation[60,68]. These changes contribute to tolerance, where increasing doses are required to achieve the same effect, and physical dependence, wherein abrupt cessation leads to withdrawal symptoms[69]. Opioids also disrupt the reward circuitry, particularly the mesolimbic dopamine pathway, reinforcing compulsive drug-seeking behavior and fueling addiction[70]. Over time, this can lead to anhedonia, emotional blunting, and executive dysfunction, undermining the user's ability to experience pleasure or make rational decisions[71]. Chronic opioid use is strongly associated with a wide range of adverse mental health outcomes, including major depressive disorder, anxiety, and heightened suicide risk[72,73]. Opioids dysregulate brain systems involved in mood and emotion—particularly those modulating dopamine and serotonin—which can lead to emotional blunting, anhedonia, and cognitive dysfunction[58].

Long-term opioid exposure has been shown to alter neurocircuitry in the prefrontal cortex and limbic system, regions critical for emotional regulation and executive function[58,74]. Epidemiological studies support these findings: individuals prescribed opioids for longer durations are significantly more likely to develop new-onset depression, even in the absence of prior mental illness[36]. More recent data underscore the high psychiatric burden among those with opioid use disorder (OUD), with a 2022 systematic review reporting comorbidity rates of 36.1% for depression, 29.1% for anxiety, and 20.9% for ADHD[75]. These mental health conditions not only increase the risk of opioid misuse and overdose but are also linked to elevated suicide rates in OUD populations[76]. Furthermore, opioid tapering itself; an act intended to reduce harm, can paradoxically increase risks for overdose and mental health crises up to two years post-reduction[77]. Despite the clear intersection of opioid use and both neurocognitive and psychiatric illness, access to integrated, evidence-based treatment remains limited, underscoring the urgent need for models to address mental health and opioid abuse in tandem. Additionally, knowing that these substances have such profound effects on adults, investigating the long-term outcomes after exposure in early development is greatly warranted.

Opioids have also been shown to impair immune function, making the body more susceptible to infections. By inhibiting the activity of immune cells such as macrophages, T cells, and natural killer cells, opioids have been shown to reduce both innate and adaptive immune responses[78-81]. This immunosuppression has been linked to increased risk of opportunistic infections, particularly in individuals with comorbidities or coexisting conditions such as HIV or hepatitis C[38]. Injection drug use, a common route of opioid administration, poses further infectious disease risk, as shared needles and other injection equipment are the primary vectors for bloodborne pathogens among people who inject drugs illicitly, and opioid-related injection practices have been linked to numerous outbreaks across the United States[82]. The rise in injection drug use during the opioid crisis has contributed to a substantial increase in HCV infections, particularly among young adults in rural areas[83]. In addition to direct transmission, opioids impair immune function by suppressing both innate and adaptive responses, potentially exacerbating disease progression in individuals already infected with HIV or HCV[84]. Moreover, opioid use disorder is associated with delayed diagnosis, reduced adherence to antiretroviral or antiviral treatment, and worse clinical outcomes for those living with these infections[85]. These compounding issues can lead to a variety of further health complications among those with weakened immune systems or exposure to infectious agents that may have been of little concern prior to opioid use.

In recent wake of the COVID-19 pandemic, opioids have shown to weaken the body's ability to mount effective antiviral responses and increasing vulnerability to respiratory pathogens like SARS-CoV-2[84]. Opioid-induced respiratory depression further compounds the risk of severe outcomes in COVID-19 patients, who often suffer from compromised lung function[86]. A large study analyzing electronic health records found that individuals with substance use disorders, especially opioid use disorder (OUD), had a significantly higher risk of contracting COVID-19 and experiencing hospitalization, mechanical ventilation, or death[87]. With the broad recognition of increased U.S. opioid overdose rates between 2020 and 2021, media, public health, and healthcare organizations have raised significant concerns over the emergence of a simultaneous COVID-19-opioid "twindemic." Research in this area has explored the possible relationships between negative externalities associated with the pandemic and/or COVID-19 public health interventions and increased risks for opioid use and overdose alongside diminished outcomes following overdose events, presenting an opportunity to highlight the consequences of compounding exposures on human health.

In summary, it is without question that both prescription and illicit opioids can cause significant harm to human health across a diversity of organ systems. However, one of the biggest remaining questions is what effects opioids may have on reproductive health, fertility, development, and offspring. Limited studies exist to determine if the effects of these substances may have long-term consequences and are often difficult to draw conclusive evidence from due to methodology, models, and design. Due to the high rate of opioid use in the United States decades ago and its steady increase on a now global scale even to this day, human exposure-relevant research is required to determine what outcomes may emerge regarding these understudied aspects of human health.

2.4 OPIOIDS AND REPRODUCTIVE HEALTH

The effects of opioids on reproductive health have been studied, although at a far lesser degree in comparison to the other effects they exhibit on other organ systems and tissues within the body. While noteworthy studies exist, they are often overshadowed by the more pressing and deadly effects of these drugs and thus overlooked by both medical professionals and the public. However, given that opioid use is highest among people of reproductive-age, and moreover is increasing, there is a need to understand how these substances interact with reproductive health and fertility.

One major problem regarding reproductive health and offspring was brought to light by the crisis within the United States: opioid exposure during pregnancy. This exposure in a particularly critical period of development presents substantial risks to both maternal and fetal health. Prenatal opioid exposure has been associated with adverse outcomes including preterm birth, low birth weight, stillbirth, and specific congenital anomalies[88,89]. In the wake of the opioid crisis, Neonatal Abstinence Syndrome (NAS), also known as Neonatal Opioid Withdrawal Syndrome (NOWS) became a massive issue and thus were critically documented to address this facet of the crisis. NAS is a condition resulting from chronic utero opioid exposure, and is often manifested by central nervous system irritability, autonomic over-reactivity, and gastrointestinal tract dysfunction[88,90,91]. Studies and reports have shown that upwards of 50% to 80% of newborns exposed to opioids develop NAS[90,92], and that incidence rate became alarmingly apparent throughout the emergence of the opioid crisis. From 1999 to 2013, the overall incidence of NAS in the states included in this report had increased almost 300%[90]. More recently, reports from Kentucky and Washington noted increased rates of NAS, surpassing the national average on statewide levels[93,94]. This relatively recent evidence indicates that opioid use remains a challenge among pregnant mothers and concerns the health and wellbeing of exposed children. While medication-assisted treatment using agents like buprenorphine or methadone is the recommended standard of care for pregnant individuals with opioid use disorder (OUD), significant barriers to treatment persist, particularly for low-income populations and racial/ethnic minorities[95]. Expanding equitable access to prenatal care, substance use treatment, and postpartum support is essential for improving outcomes for both mothers and their children.

It was with this tragic abundance of children born with NAS over the last few decades that led to researchers turning their attention towards the potential long-term developmental outcomes and health concerns of opioid exposure. Studies have shown that children born with a history of in utero opioid exposure demonstrate increased risk for neurodevelopmental delays, including cognitive impairment, motor dysfunction, and behavioral disorders such as attention-deficit/hyperactivity disorder (ADHD)[96-99]. Animal studies make up the majority of this research due to both ethical standards and the given benefits of animal models, but notably there are many differences among model animals and humans regarding reproduction and exposure responses. Of the few human studies that exist, however, many of them remain inconclusive due to study design, cohort availability, and a lack of structured and robust methodology. In an effort to better understand this connection between long-term developmental outcomes and exposures, large-scale monitoring methods are emerging, such as the Environmental influences on Child Health Outcomes (ECHO) program[100], an initiative to identify the link between these exposures and influences on the health and development of children, are required to better understand what long-term effects opioids may have on development. Additionally, robust and dynamic human in vitro models offer promise in observing these more complex, long-term complications that may arise post opioid exposure.

Opioids, both endogenous and exogenous, have been implicated to have a range of profound effects on the endocrine system, which in turn can lead to further complications throughout the body. The endocrine system consists of a network of glands such as the pituitary, thyroid, and adrenal glands, and plays a crucial role in tightly regulating various physiological processes including growth, metabolism, and reproduction[101]. Even acute opioid use has been shown to induce endocrine disruptions, while chronic use having the potential to disrupt the hypothalamic–pituitary–gonadal (HPG) axis, leading to suppression and potential long-term damage[102]. Although results are often conflicting and differ among study design and species, a body of scientific evidence has shown that opioids generally increase growth hormone (GH) and prolactin (PRL), and decrease luteinizing hormone (LH), testosterone, and estradiol[103]. In conjunction, chronic opioid use suppresses the HPG axis resulting in secondary testosterone deficiency known as Opioid-Induced Androgen Deficiency (OPIAD)[104].

The HPG axis is of particular concern regarding its connection to reproductive health. This axis involves the hypothalamus, pituitary gland, and gonads, and regulates the production of sex hormones such as testosterone, estrogen, and progesterone[105.] Opioids have been shown to disrupt the normal functioning of the HPG axis, leading to alterations in reproductive hormone levels and downstream effects on reproductive function[106]. In women, chronic exposure can result in irregular menstrual cycles, reduced fertility, and in some cases, opioid-induced hypogonadism, which reduces estrogen and progesterone levels and may contribute to infertility or early menopause[107-109]. Chronic opioid use has been associated with reduced levels of testosterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH), which are essential for spermatogenesis and overall reproductive health in men[110]. Existing literature suggests that chronic opioid use may adversely affect various aspects of male reproductive function, including sperm quality, sperm count, and sexual function[111], but further research is needed to elucidate the mechanisms underlying these effects and their significance long-term.

With a growing body of evidence highlighting dangers of opioid exposures on reproductive health and development, including potential long-term repercussions, there are concerns that these impacts may potentially be transmitted to subsequent generations.

Due to the substantial percentage of children born with NAS over the past few decades, most research has focused on female exposures during pregnancy, while male opioid use and the impacts on reproductive outcomes have been understudied and unappreciated. In general, there is an absence of literature regarding male reproductive contributions in association with potential environmental exposures, in part due to the abundance of research on both pregnancy and female reproduction[112], but also due to the challenges in studying both impacts and mechanisms from a male or paternal origin[113].

It was not until recent years that reports emerged with evidence pointing towards the possibility of paternal influences on the health of offspring. Specifically, research has shown that opioid use, even before conception, influences the reactivity to opioids of the progeny and the following generations, likely through epigenetic mechanisms[114]. Regarding epigenetics, another study increased DNA methylation levels in blood samples at the *OPRM1* promoter region, the gene responsible for encoding the mu-opioid receptor, in opioid addicts when compared to controls[115]. Interestingly, preconception paternal opioid exposure was found to be associated with significantly higher anxiety-like behavior as well as enhanced voluntary consumption of morphine in morphine-derived rat offspring compared to controls[116]. A more recent study showed that paternal morphine exposure impacted the reward sensitivity of morphine in male, but not female rat progeny as assessed using self-administration[117]. One analysis determined impaired inhibitory control as well as exhibiting delayed learning and impulsive behavior in male rat offspring[118]. A recent human study found that regular paternal opioid use was independently associated with increased odds of overweight or obesity in offspring[119]. These findings add to the growing concerns that suggest the potential implications of paternal preconception opioid exposure, namely via epigenetic modifications that could occur during the critical reprogramming period of the male germline, and thus impact subsequent generations.

Considering that opioid use, both prescription and illicit, remains high and continues to rise on a now global scale, and men of reproductive age make up the largest proportion of opioid users, it is critical to understand how opioid use may lead to deficits, both short and long-term, in male reproductive health, as well as potentially impact the health of offspring. While paternal preconception exposures were often disregarded and thus understudies due to the thought that they were unable to lead to meaningful impacts in offspring, this emerging evidence alongside the rise in opioid use demands critical scientific evaluation.

2.5 THE MALE REPRODUCTIVE SYSTEM

The male reproductive tract is a complex and dynamic system governed by the hypothalamic-pituitary-gonadal (HPG) axis. Post puberty, gonadotropin-releasing hormone (GRh) is produced and secreted by the hypothalamus, then acts upon the anterior pituitary gland to produce both follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Together, these hormones travel to the testis and act upon somatic cells that reside within. LH acts upon Leydig cells, facilitating the production and secretion of testosterone. FSH works in conjunction with testosterone and Sertoli cells to facilitate spermatogenesis and thus spermiogenesis[120]. This system forms an interconnected and tightly regulated feedback loop that is critical for male reproductive functionality, as it is consistent throughout the adult lifespan. It is critical to note that any substance that may interact with the endocrine system could disturb these hormones, leading to alterations not only in endocrine function, but also in male reproductive health and thus fertility.

Spermatogenesis is both a complex and organized process that occurs cyclically throughout the reproductive life of male organisms[120,121]. Male gametes are produced from spermatogonial stem cells (SSCs) through several stages of mitotic and meiotic cell divisions. These SSCs sit along the outside of the blood-testis barrier (BTB), and both divide to maintain the SSC pool retained throughout adult life, as well as progressing through the immune-privileged barrier as they differentiate. The progenitor cells divide mitotically as they become differentiated, thus undergoing meiosis. These differentiated SSCs undergo mitosis to produce primary spermatocytes, which then proceed to enter meiosis. Following this, the cells with undergo two meiotic divisions to produce secondary spermatocytes and round spermatids. Round spermatids undergo morphological transformations to become mature spermatozoa, involving nuclear condensation, formation of the acrosome and tail in the process known as spermiogenesis[122].

Endogenous Opioids within the Male Reproductive System

Recent evidence has emerged suggesting that the endogenous opioid system plays a role in modulating male reproductive function. Studies have shown the presence of endogenous opioid peptides (EOPs) alongside their respective opioid receptors in different organs and tissues within the male reproductive system indicates that EOPs likely participate in some role of reproductive homeostasis[123]. It is well known that opioids interact with a variety of organ systems, including the endocrine system and the HPG axis. In this regard, opioids have generally been shown to decrease not only LH but also testosterone and estradiol, which have effects on testicular function[103,124-126]. This interaction is suspected to play a role in the regulation of the HPG axis, however, exogenous opioid use has been shown to induce hypogonadism alongside decreased libido and erectile dysfunction in men chronically abusing opioids, as well as infertility[124]. These findings collectively indicate that EOPs can regulate reproductive function by inhibiting the secretion of GnRH at the level of the central nervous system[127,128]. Studies have demonstrated not only the presence of EOPS in different testicular cell types, but have also confirmed the presence of all three respective opioid receptors (MOR, DOR, and KOR) in rat testes[129-132]. In the testis, EOPs have been shown to be mainly synthesized *de novo* by Leydig cells and produce an inhibitory paracrine effect on Sertoli cells, including inhibiting their proliferation and differentiation[133,134]. Together, the expression of both EOPs and their respective receptors in the male reproductive tract indicate the potential for both the indirect and direct involvement of endogenous opioids on male reproductive function. While exogenous opioid use has been shown indirectly impact male reproductive health, the direct effects on the male reproductive tract remain obscured.

Recent studies have also discovered the presence of the three primary opioid receptor types, although at differential expression levels and localization patterns, specifically in the germ cells[135,136]. These findings suggest that opioid receptors may be not only expressed during spermatogenesis but are important in either sperm cell maintenance or function. These studies provide credibility that opioids are important in some aspect regarding either spermatogenesis or maintenance of the testicular microenvironment, and that alterations in opioid levels in this specialized and regulated process may alter critical aspects of male reproductive health.

With the male reproductive process being highly complex and sensitive, it can be said that any disruptions or exposures, at any critical point during this process, have the potential to induce negative consequences. Given the importance of the endogenous opioid system and its implications in male reproduction, exogenous opioid use has the potential to lead to severe disruptions including impaired fertility, hormone production, alterations in sperm production and development, semen parameters, and offspring health.

Windows of Susceptibility

During male reproductive development, there are multiple critical windows of susceptibility during which environmental exposures, including pharmaceutical agents, may interfere with the proper formation and function of germ cells. These windows represent periods of heightened cellular sensitivity, during which disruptions to hormonal signaling or cellular differentiation pathways may have long-lasting implications for adult fertility. One of the most well-characterized of these occurs *in utero*, where early developmental perturbations have been associated with a spectrum of congenital anomalies, including cryptorchidism, hypospadias, impaired spermatogenesis, poor semen quality, and increased risk of testicular germ cell tumors[137,138]. Collectively, these conditions are encompassed under the framework of testicular dysgenesis syndrome (TDS), which is thought to originate, at least in part, from disruptions to fetal androgen exposure or action[137].

While certain cases of TDS have been linked to underlying genetic defects[139], accumulating evidence suggests that environmental and pharmaceutical exposures during sensitive developmental windows may also contribute[140]. Importantly, the fetal period is not the only window during which germ cell development is vulnerable. Postnatal stages, including the neonatal period, mini puberty (a transient activation of the hypothalamic-pituitary-gonadal axis in early infancy), peri puberty, and puberty itself, each involve critical endocrine and morphological changes that are essential for the establishment of a functional spermatogenic lineage[141]. Disruption of androgen signaling during any of these windows has the potential to impair Sertoli cell maturation, Leydig cell steroidogenesis, or peritubular myoid cell function, all of which are essential for maintaining the spermatogenic niche.

Pharmacological agents that interfere with these pathways may lead to delayed or abnormal germ cell development, impaired spermatogenesis, and subfertility or consequentially infertility in adulthood. This concern is particularly relevant given the increasing prevalence of pharmaceutical use, including opioids, among adolescent and adult males of reproductive age. In addition to their host of other negative effects, these compounds may also exert direct cytotoxic or epigenetic effects on developing germ cells, further compounding the risk to reproductive health through these highly susceptible timepoints.

2.6 CHALLENGES AND INNOVATIONS IN STUDYING INFLUENCES ON MALE REPRODUCTIVE HEALTH

In recent years, the rate of both legal and illicit substance use among men of reproductive age in the United States has increased substantially. Nearly 70% of men between the ages of 18 and 44 report taking either prescription or over-the-counter medications[142]. In addition, 16.7% Americans aged 12 and older battled a substance use disorder in 2023[143]. These trends in substance us are occurring alongside delayed fatherhood, with nearly 25% of men in developed countries are now having their first child after the age of 40[144]. As paternal age increases, so does cumulative exposure to a host of environmental and lifestyle influences, raising important questions about their impact on reproductive function and offspring health.

Despite the significant contributions of men when it comes to reproduction and fertility, male reproductive health is frequently underemphasized in clinical settings and understudied in research. Impairments in semen quality and fertility are not only reproductive concerns but have also emerged as potential biomarkers of overall health, linked to chronic illness and even reduced life expectancy[1,145-147]. Nevertheless, these indicators are often disregarded when healthcare providers prescribe medications, rarely considering the patient's reproductive intentions or the potential consequences of pharmacological exposures on fertility. This gap in clinical practice is particularly concerning given the established mechanisms by which medications can disrupt male reproductive function, whether through hormonal interference, direct gonadal toxicity, or alterations in sperm production and sexual health. Alarmingly, only a fraction of medical providers and health specialists are aware that certain medications may pose these risks[142], underscoring the urgent need to integrate reproductive health evaluations into general medical care for men.

A contributing factor to the lack of both scientific and public knowledge in this area largely stems from an absence of testing requirements. In fact, reproductive toxicity in men was not a standard requirement in drug testing until 2011, when the U.S. Food and Drug Administration (FDA) introduced guidelines for preclinical screening evaluations that including the effects of drugs on hormone regulation, semen parameters, mating behavior, and overall fertility[148]. However, most drugs currently in use were approved prior to this regulation, meaning their reproductive risks are often undocumented or inadequately described in official labeling[149]. Opioids, for example, are widely prescribed and frequently abused, yet their potential to harm male fertility is still underappreciated both in medical literature and practice.

Studying the reproductive effects of substance abuse adds further complexity. Individuals who misuse drugs, such as opioids, stimulants, or cannabinoids, often face socioeconomic challenges, mental health comorbidities, and inconsistent healthcare access, all of which complicate longitudinal research[150]. Furthermore, ethical constraints limit the ability to conduct experimental studies in human subjects, especially when investigating outcomes related to conception, pregnancy, and child development[91]. Substance use is also often underreported[151], making it difficult to establish clear causal relationships between exposure and reproductive outcomes. Direct evidence from a particular drug in the case of substance abuse can be difficult to parse out among other factors involved, particularly in terms of cohort studies. These methodological hurdles create significant barriers to understanding how substance misuse affects fertility, especially in men, where reproductive health is less frequently studied or prioritized. In contrast, a vast body of research exists on drug effects in women, particularly during pregnancy. This disparity is driven not only by the biological connection between mother and child but also by the relative accessibility of maternal-fetal endpoints. In men, reproductive effects are more diffuse, delayed, and difficult to measure, especially in the context of environmental and behavioral confounders such as substance use. Bridging this gap will require targeted research, increased clinical awareness, and policies that prioritize reproductive health in both sexes.

The majority of our understanding of chemical and drug interactions on reproductive health, including male reproductive health and spermatogenesis, originates from the use of animal models[138,152]. While there are many types of models that offer niche advantages, rodents, particularly mice and rats, are the most abundantly used *in vivo* models in reproductive toxicology[153,154]. These species are favored in biomedical research due to their small size, minimal maintenance requirements, and short gestational periods. They produce large litters and reach sexual maturity quickly, making them efficient for studying reproductive biology[153]. However, despite these advantages, fundamental species-specific differences in reproductive physiology limit the direct translation of these findings to human male fertility.

First, significant kinetic and biological differences exist between species. For example, mice possess a relatively small spermatogonial stem cell (SSC) pool, representing only about 0.3% of their total spermatogenic cells. Non-human primates have a larger SSC pool at approximately 4%, whereas humans exhibit a substantially higher proportion, around 22%[155]. After division, SSCs give rise to undifferentiated cells that undergo multiple rounds of transit-amplifying mitoses before entering meiosis. The number of these mitotic divisions determines sperm production capacity. In mice, there are approximately 12 divisions; in non-human primates, 8; and in humans, only 5. This results in dramatic variation in daily sperm production per gram of testis: about 40 million in mice, 41 million in non-human primates, and just 4.4 million in humans[155].

Differences are not only limited to cell population sizes. Additionally, the duration of spermatogenesis also varies greatly among mammals. In mice, the process of spermatogenesis takes roughly 35 days, while in humans it spans approximately 64 days[156-158]. Furthermore, there are key disparities in pharmacokinetics, such as drug absorption, distribution, metabolism, and accumulation, especially within the male reproductive tract[158,159]. Furthermore, animal models often require high doses of test substances to elicit a toxic effect, which can limit the applicability of those results to humans. Thus, measurements such as sperm concentration, motility, and morphology may not accurately reflect actual fertility potential[160], as these parameters are highly variable even among fertile men. Finally, it is important to note that many animal studies typically focus on hormone levels and gross morphological changes to evaluate reproductive toxicity but rarely assess molecular or subcellular alterations. This lack of resolution, combined with interspecies variation in drug sensitivity, hinders the extrapolation of findings to human populations and real-world exposures.

Consequently, these models, while valuable, have inherent limitations that challenge their clinical relevance. Human health outcomes can be investigated more directly through pharmaceutical and observational studies provided they are within ethical bounds. Ideally, the impact of medications on male fertility would be examined using large, randomized, double-blinded, placebo-controlled clinical trials. Yet there are a miniscule number of studies that match these criteria in the real world. When conducted, they often include too few male participants to detect meaningful effects on male reproductive health and fertility. Properly powered observational studies may offer more clinically relevant insights, but they are still limited by factors such as heterogeneity in drug class effects and interindividual variability in drug response.

Another issue involving the study of drug use is that drugs within the same pharmacological class can produce different reproductive outcomes, and personal factors, such as genetics, environmental exposures, and co-administered medications, can influence how a drug affects fertility. These challenges underscore the need for more refined, human-relevant models to assess reproductive toxicity. Emerging *in vitro* systems using human cells provides a promising platform for better evaluation. Such models can offer valuable data to healthcare providers, potentially improving prescribing practices and reducing unintended reproductive harm.

Due to the limitations of animal models and the increasing need of more comparable human studies, there has been a growing push to develop alternative approaches to animal testing wherever possible. This movement has received staunch support from national regulatory agencies such as the U.S. Food and Drug Administration (FDA) and the U.S. Environmental Protection Agency (EPA), both of which have prioritized efforts to reduce animal testing. These agencies have encouraged laboratories and academic institutions to develop and adopt alternative methods and testing strategies. In 2019, the EPA announced that it would reduce its requests for and funding of animal studies by 30% by 2025, with the ultimate goal of eliminating such testing by 2035. Similarly, the FDA has launched programs to foster the development of alternative testing technologies[161]. These efforts align with the principles of the “Three Rs”: Replacement, Reduction, and Refinement, which involves substituting animal-based protocols with non-animal methods, minimizing the number of animals used while maximizing data quality, and refining procedures to reduce the suffering of any animals still involved[162].

While animal models have historically been the cornerstone of toxicological assessment, both clinical trials and epidemiological cohort studies have offered promise in learning of environmental exposures and toxicological effects. Yet even these studies have their drawbacks, mainly being reliant on a population of people subject to accidental or unintended exposure. Not only can this be difficult to capture and study from a scientific perspective, but it can include numerous additional external factors, as well as expensive and time-consuming. Given the need for novel, robust models that can aid in determining toxicological effects, there has been a push to adopt *in vitro* cell culture methods to address the current state of human health research.

Fortunately, scientists were already developing innovative ways to meet this new standard. Over the last few decades, research has increasingly leveraged stem cell–based models to explore human biology. As of current, researchers can purchase stem cells from NIH-approved biomedical companies such as Stanford Medicine or WiCell. These include both human embryonic stem cells (iESC) and induced pluripotent stem cell (iPSC) lines; many of which are engineered to model specific diseases or genetic mutations and come from a diverse array of genetic backgrounds. The availability of these cells allows for scientific consistency and reproducibility across studies as well as uniquely allowing for cell developmental studies. In tandem, the use of patient-derived iPSCs has become more prevalent in both medicine and research and holds great potential for testing drug safety and efficacy *in vitro* before administering treatments to human subjects[163]. Together, these new cell-based systems allow for toxicological and pharmacological evaluations across various genetic backgrounds, enabling researchers to identify sensitive populations while reducing confounding variables and minimizing reliance on animal models.

Stem cell–based models are valuable not only in drug development and safety assessment but also in screening for potential environmental toxicants[13,14,164]. As such, these alternative approaches offer an efficient, scalable, and human-relevant strategy to evaluate the potential adverse effects of substances, safer pharmaceuticals, and protecting public health.

2.7 STUDYING THE EFFECTS OF OPIOIDS USING AN *IN VITRO* BLOOD-TESTIS BARRIER MODEL

In the late 19th and early 20th centuries, scientists were looking into examining tissues within the bodies of animals. By injecting dyes into the body, scientists found that both the brain and interestingly the seminiferous tubules of the testes remained unstained. This early evidence suggested the presence of barriers that separated blood from these privileged organs[165]. However, it was not until the early 1960's when Chiquoine was investigating cadmium-induced injury to the testis coined the term blood-testis barrier[166]. A few years later, scientists began uncovering the physiological role of the blood-testis barrier (BTB), noting the restriction of passage of substances from the blood into the seminiferous tubules, and that the barrier's permeability was facilitated and formed by tight junctions between Sertoli cells [167,168]. Now, it is understood that this blood-testis barrier is one of the tightest blood-tissue barriers in the body, and critical for male reproductive success.

Sertoli cells, also known as nurse cells, assist in the proliferation differentiation and maturation of mature male germ cells. These somatic cells make up the blood-testis barrier, a specialized and dynamic structure that separates the seminiferous epithelium into apical, adluminal, and basal compartments in the testes. The BTB has multiple functions critical to the facilitation of spermatogenesis. First, the blood testes barrier acts as a gateway, regulating the movement of nutritional substances, like amino acids, vital molecules, like hormones, as well as exogenous substances, such as pharmaceuticals and toxicants into the adluminal compartment where spermatogenesis and spermiogenesis occur[169]. This is due to the blood vessels capillaries and lymphatic vessels residing in the interstitium between the seminiferous tubules without running through them. This results in the blood testes barrier having to regulate the transcellular in paracellular transport of these substances across the barrier into the apical compartment. Second, the blood testes barrier is crucial to the seminiferous epithelium by maintaining cellular polarity[170]. In addition to the localization of Sertoli cell nuclei near their basal compartment, the blood testes barrier regulates cellular events that occur during spermatogenesis by providing segregated areas between cells undergoing spermatogenic renewal, proliferation, and differentiation[169,171,172]. The spermatogonial stem cells, undifferentiated and differentiated, spermatogonia, and preleptotene spermatocytes are located within the basal compartment, while the zygotene, pachytene, and depleting spermatocytes specs secondary spermatocytes spermatids as well as spermatozoa, located within the adluminal compartment of the seminiferous tubules.

Finally, the BTB creates an immunological barrier by suppressing an immunological response to auto antigens residing in the germ cells undergoing meiosis. This blood test is barrier function contributes to the immune privileged status of the tastes to avoid production of anti-sperm antibodies which would then lead to infertility via macrophage invasion[173]. Additionally, the Sertoli cells contribute to maintaining the immune-privileged state of the testes by secreting immunosuppressive molecules to block an immune response to the auto antigens in the differentiating germ cells[174]. Although many of these biomolecules remain to be uncovered, they are speculated to consist of both prostaglandins and cytokines. Thus, it is possible that pharmaceuticals or environmental toxicants with the ability to alter the immunosuppressive properties of the Sertoli cells may have the ability to contribute to disease.

Uniquely, the BTB is composed of four different cellular junctions, which include tight junctions, gap junctions, desmosomes, as well as ectoplasmic specializations, each of which playing a key role in the structure and permissibility of tis barrier[171,175]. Tight junctions work via managing the flow of proteins and lips between the apical and basolateral compartments as well as restricting the flow of both solutes and large molecules between the paracellular space. Gap junctions are channels that form cell-to-cell connections, allowing the flow of small molecules, ions, and metabolites[176]. Desmosomes also involve cell-to-cell contact and are found at the Sertoli-spermatid interface and between adjacent Sertoli cells at the BTB, serving as anchoring junctions[169,177]. Ectoplasmic specializations are specific adhesion junctions within testes microenvironment and consist of two types, with the first being basal ectoplasmic specializations that reside between Sertoli cells, and the second being apical ectoplasmic specializations between Sertoli cells and elongating spermatids[177]. Together these junctional proteins coexist together to form one of the tightest and highest regulated blood tissue barriers in the human body.

However, despite this rigidity, the BTB must also be dynamic to facilitate the movement of the preleptotene spermatocytes from the basal to the apical compartment as they differentiate into zygotene and pachytene primary spermatocytes. This unique junctional coordination regulates the temporal opening and closing of the blood testes barrier to facilitate the movement of the preleptotene spermatocytes in the adluminal compartment[169,172,177,178]. With the BTB being such a dynamic and critical aspect of the male reproductive system, it is important to note that any perturbations from medications or environmental toxicants that induce testicular injury through interactions with the blood testes barrier can result in subsequent damage to the developing germ cells and cause damage to male reproduction as well as male infertility.

An In vitro Blood-Testis Barrier Model

Our understanding of testicular physiology and toxicology can be attributed to utilizing *in vitro* models, specifically via both primary cells and cell culture methods; both of which are available via several commercial biotechnology providers. These companies supply validated and functionally characterized primary cell types derived from various human tissues, including skin, liver, kidney, and reproductive organs, for use in academic and pharmaceutical research worldwide. This accessibility allows researchers to use standardized, reproducible human cell lines to examine how environmental exposures may alter testicular physiology, particularly BTB function, before advancing to more complex and resource-intensive *in vivo* or epidemiological studies, in which ethical concerns arise.

In the field of male reproductive biology, *in vitro* models of the BTB are require using Sertoli cells, the somatic component of the seminiferous epithelium responsible for nurturing developing germ cells and forming the structural basis of the BTB. Sertoli cells, when cultured at high density on an extracellular matrix, can form polarized epithelial monolayers that closely mimic the BTB both structurally and functionally. These cultures give rise to ultrastructural features including tight junctions, gap junctions, ectoplasmic specializations, and desmosomes, all of which being key aspects in the establishment and maintenance of the BTB[165,178]. This model system allows for detailed investigation of the molecular interactions that regulate junction formation and remodeling, as well as the dynamic responses of the BTB to hormonal cues or toxic exposures.

In recent years, *in vitro* BTB models have been employed to study the impact of several environmental toxicants. Agents such as cadmium, ethanol, and even COVID-19-related inflammatory factors have been shown to compromise BTB function in these systems, resulting in impaired barrier integrity and altered Sertoli cell function[179]. Importantly, a recent study found that opioid dependance and withdrawal increased oxidative stress and inflammatory response in the Sertoli cells of mice[180]. These findings underscore the utility of Sertoli cell-based models in dissecting the cellular and molecular mechanisms of testicular dysfunction. With continued refinement, such models hold promise for screening environmental and pharmaceutical compounds that may affect male fertility and for identifying potential therapeutic strategies to preserve reproductive health.

The ability for extracellular molecules and exogenous substances to move across the epithelial barrier can happen either paracellular or transcellular movement, with the former being between cells, and the latter being through cells directly[181]. Paracellular movement involves the passive diffusion of molecules via regulation by tight junctions. Transcellular movement is governed by specific transporters along either the apical or basolateral plasma membranes[182]. In turn, a compound's hydrophilic or lipophilic nature plays a role in how they cross barriers, hydrophilic crossing paracellularly and lipophilic crossing transcellularly. In the case of opioids, namely fentanyl, transcellular transport is the primary pathway in which these drugs cross barriers to exhibit their effects, namely the blood brain barrier. Thus, an *in vitro* BTB model provides a novel and meaningful tool in assessing the impact of opioids on the BTB's integrity and functionality from these junctions in a controlled and reproducible manner.

To assess barrier disruption or dysfunction following exposure to xenobiotics, it is essential to quantify both the functional permeability and the structural integrity of the barrier. Two widely used and complementary techniques for this purpose are transepithelial electrical resistance (TEER) measurement and paracellular flux (dye-flux) assays. In the *in vitro* BTB model, Sertoli cells are cultured on a porous semi-permeable membrane, where they establish junctional complexes that replicate the barrier properties of the *in vivo* BTB. TEER provides a quantitative measure of the ionic conductance across the Sertoli cell monolayer. Electrodes are placed in the apical and basal chambers of the Transwell insert, and the resistance to electrical current is recorded. A higher resistance indicates greater tight junction integrity, whereas a decline in TEER suggests increased paracellular permeability or barrier disruption. TEER is widely accepted as a sensitive, real-time, and non-invasive indicator of epithelial barrier function.

The paracellular flux assay, on the other hand, provides a complementary measure of barrier permeability using fluorescent tracer molecules. In this assay, probes of defined molecular weights are added to the apical chamber, and aliquots are collected from the basal chamber at various time intervals. The concentration of fluorescent signal is measured using a plate reader, allowing for the quantification of molecular diffusion across the barrier. These techniques have been applied in other studies looking at the effects of other exposures on epithelial barriers[183] and offer a robust and reproducible means to monitor dynamic changes in BTB permeability.

While emerging technologies like testicular organoids offer exciting possibilities for modeling spermatogenesis *in vitro*, TEER and dye-flux assays remain cost-effective and accessible methods, particularly for toxicological screening. These assays are well-established in the literature, relatively simple to execute, and provide meaningful insight into how environmental or pharmaceutical agents may disrupt the BTB.

In total, a Sertoli cell-based *in vitro* model combined with unique barrier-assessment assays allow for the scientific exploration of not only how toxicants cross the BTB, but also how such exposures may affect downstream processes critical to male fertility such as meiosis and germ cell differentiation. Chapter 3 encompasses the use of these assays alongside a human *in vitro* BTB model in order to better understand the direct effects of opioids on male reproductive health. In conjunction with gene expression analyses and using clinically relevant doses of the potent opioid Fentanyl, this study aims to assess the BTB and its most critical cells, Sertoli cells, to better understand the impacts these substances have, and bring that knowledge to both the scientific community and the public.

2.8 STUDYING THE EFFECTS OF OPIOIDS USING AN *IN VITRO* SPERMATOGENESIS MODEL

In recent years, great progress has been made across a broad range of reproductive technologies. With the rise in infertility alongside other health concerns, innovative models and techniques are required to better reconstruct real-world exposures, toxicities, and further uncover the mechanisms of biology. When approaching the study of male reproduction, *in vitro* spermatogenesis has become a critical tool in modeling these influences. Furthermore, it has shown promise in terms of aiding some of the complex, known issues involved with male infertility[184].

The *in vitro* spermatogenesis model developed by Easley and colleagues brings a valuable platform to the field of reproductive biology[185]. This model provides an excellent way to directly evaluate the impact of environmental exposures such as chemicals and pharmaceuticals on the male reproductive system. As previously described, there are a multitude of known substances that have the capability to disrupt male reproductive health by acting as endocrine-disrupting chemicals (EDCs), interfering with the hypothalamic-pituitary-gonadal (HPG) axis, impairing spermatogenesis, or altering sperm count and semen parameters. Although there is evidence that opioids can influence various aspects of male reproductive function, the underlying mechanisms of opioid-induced reproductive toxicity, particularly their direct effects on human spermatogenesis and the gametes, remain poorly understood. Due to these unknowns and the issues involving these effects in humans, an *in vitro* human spermatogenesis model is uniquely poised to study these effects in both an ethical and relevant fashion.

Further regarding this, there is great discussion on the topic of epigenetics, and whether opioid use may leave lasting alterations in the sperm epigenome and thus be potentially inheritable by offspring and persist in the next generation. A growing body of evidence indicates that other environmental factors can induce epigenetic modifications in sperm, potentially affecting male fertility and offspring health. Notably, one study found that paternal high-fat diets have been associated with altered DNA methylation in genes related to metabolism and insulin signaling, leading to metabolic dysfunction in offspring[186]. Similarly, chronic psychological stress has been shown to alter small non-coding RNAs in sperm, such as microRNAs, which regulate gene expression in early embryos and have been implicated in neurodevelopmental changes in progeny[187]. Exposure to EDCs, like bisphenol A (BPA) and phthalates has been linked to changes in DNA methylation and histone modifications in sperm, particularly in genes involved in reproductive function and embryonic development[188]. Tobacco, one of the most commonly used substances worldwide, has been associated with increased sperm DNA fragmentation and aberrant methylation patterns in imprinted genes, potentially compromising fertility and increase the risk of disease in children[189]. These findings not only underscore the sperm epigenome's sensitivity to environmental exposures and highlight a potential transmission of paternal lifestyle and environmental conditions into future generations.

As previously described, spermatogenesis is a highly coordinated developmental process that transforms diploid spermatogonial stem cells (SSCs) into mature haploid spermatozoa[20,112,120,190,191]. This elaborate process, however, also involves extensive epigenetic reprogramming, including DNA methylation, histone modification, and chromatin remodeling, which together alter gene expression patterns critical for germ cell differentiation. During spermatogonial differentiation, histone-to-protamine replacement compacts the chromatin and facilitates the streamlined shape of mature sperm. Prior to this, histone modifications are carefully regulated to allow or restrict transcription of germline-specific genes[192,193]. Among the epigenetic mechanisms, DNA methylation is one of the most extensively studied. This modification plays a crucial role in regulating gene expression, maintaining genomic stability, and guiding cellular differentiation during development. In early germ cells, global DNA demethylation resets the epigenetic landscape, followed by *de novo* methylation to establish a male-specific pattern. This wave of epigenetic reprogramming is essential for erasing somatic epigenetic marks and preventing transgenerational epimutations. Importantly, errors in this process can result in infertility, embryonic arrest, or the transmission of aberrant epigenetic marks to offspring, with long-term consequences for health and development[141,189]. This underscores the sperm epigenome's dual role: as a marker of male reproductive health and a vector of heritable environmental information.

Studies have uncovered that both endogenous opioids and their respective receptors have been located within the male reproductive system, including gametes, and are believed to play some role in the process of spermatogenesis[103,123,127,130,133,135,136,194,195]. Specifically, exogenous opioids have been shown to alter functions of the HPG axis and thus lead to changes within the male reproductive tract, usually in the form of hypogonadism, however, few but noteworthy studies have also shown changes in sperm [115,196-198], While these studies shed light on the possibility of opioids attributing to consequences in sperm function and health, and moreover epigenetic changes in the germline, they are few in number, and difficult to reproduce, indicating a need for further examination.

Using an *in vitro* human spermatogenesis model allows for the unique ability to mimic opioid use and directly assess epigenetic alterations in sperm. With this model, multiple genetic lineages can be screened across clinically relevant doses of opioids throughout the differentiation process in not only an ethical, but an efficient method. Being highly relevant to the current worldwide opioid crisis and epigenetic influences, this model provides an important tool capable of examining changes that may arise in the real-world and provide critical knowledge that may assist in a clinical manner regarding male reproductive health, infertility, and inheritable consequences.

Chapter 4 encompasses this study in detail, focusing on the effects opioids have on male germline cell epigenome. Specifically, examining fentanyl's effects on the DNA methylation patterns in the *OPRM1* promoter region of round haploid spermatids. The *in* *vitro* spermatogenesis model allows for the differentiation of pluripotent stem cells into round haploid spermatids, which represent the final post-meiotic stage of spermatogenesis. Notably, although not yet fully mature spermatozoa, they feature a complete haploid genome that possesses all the genetic and epigenetic information necessary for embryonic development. This includes DNA sequences, DNA methylation patterns, retained histones, and sperm-borne RNAs that can influence zygotic gene regulation after fertilization[193,199]. Furthermore, the *OPRM1* gene is heavily implicated in modulating addiction and attributing to other neurological outcomes, but its presence and function is the male reproductive system remains somewhat unknown. Studies have implicated the potential for substance abuse to be heritable through epigenetic alterations in the male germline[117,200], however these studies utilized animal models with notable differences to humans regarding spermatogenesis. Therefore, an *in vitro* human spermatogenesis model in conjunction with epigenetic detection assays gives a unique and relevant method in assessing epigenetic changes from environmental origins on developing sperm.

Future studies could use this model to observe the effects of other opioids, including various mixtures with other drugs to better simulate a real-world scenario of drug and substance abuse, as well as encompass more genome-wide examinations to get a better insight into the epigenetic landscape changes brought on by exposures. An *in vitro* human spermatogenesis model can provide a new standard to assess potential consequences in reproductive health, how pharmaceutical compounds are assessed, and provide knowledge that can aid human health. In conjunction with the *in vitro* blood-testes barrier model, these unique tools and methods can provide much-needed understanding to the effects of substance abuse on critical aspects of the male reproductive tract and what consequences that may have for fertility, health, and offspring.

CHAPTER 3

INVESTIGATING THE IMPACTS OF EXOGENOUS OPIOIDS ON THE BLOOD-TESTIS BARRIER

3.1 INTRODUCTION

Over the last several decades, opioid use and abuse has been on the rise, particularly within the United States, however now developing into a multinational crisis[29,30]. Although prescription rates have declined, illicit opioids such as fentanyl have emerged, being of particular concern due to their abundance and potency and the high percentage of use and overdose related mortality rates in North America[50]. Evidence suggests that men, particularly those of reproductive age, make up a large majority of opioid users[52], and is increasing. Opioid use, both short and long-term, has been shown to alter reproductive function and have negative impact on male fertility. Reduced sperm counts, motility, and hypogonadism have been documented in the literature[102-104,110]. It is implied that exogenous opioid use alters the endocrine system, and thus the reproductive system via interaction with the HPG axis. However, there is little known about the direct effects of opioids within testis, particularly within the blood-testis barrier (BTB). There is evidence to suggest that the endogenous opioid system plays a role in male reproductive health and spermatogenesis via direct opioid and receptor interaction within the reproductive tract, however, more research is warranted to determine how these potent opioids interact with Sertoli cells which are critical for BTB structure, function, and spermatogenesis. Therefore, elucidating the direct impacts of exogenous opioids on Sertoli cells, and thus the BTB, without involvement of the endocrine system, is warranted.

3.1.1 The Blood-Testis Barrier

The blood-testis barrier (BTB) is a highly specialized and dynamic structure essential for normal spermatogenesis and overall male reproductive function. Formed primarily by tight junctions between adjacent Sertoli cells within the seminiferous epithelium, the BTB physically divides the seminiferous tubules into basal and adluminal compartments[167-169,171]. This compartmentalization is crucial for creating an immune-privileged environment that protects developing germ cells, especially those undergoing meiosis and spermiogenesis, from autoimmune attack. As germ cells progress through spermatogenesis, they express novel surface antigens not recognized as "self" by the immune system; without the BTB, these cells would be vulnerable to immune-mediated destruction. In addition to immune protection, the BTB regulates the passage of ions, nutrients, hormones, and signaling molecules into the adluminal compartment while preventing the entry of blood-borne toxins or pathogens that could disrupt spermatogenic processes[169,173-175,178]. Disruption of the BTB, whether by toxicants, inflammation, or disease, can lead to impaired germ cell development, decreased sperm quality, and ultimately infertility. Moreover, the integrity of the BTB is dynamically regulated throughout the spermatogenic cycle, requiring precise coordination of cell junction remodeling and Sertoli cell function. For these reasons, the BTB is considered one of the most critical components in maintaining a functional testicular microenvironment and ensuring male fertility.

3.1.2 BTB Disruption by Environmental and Pharmaceutical Agents

Multiple environmental and pharmaceutical agents have been shown to disrupt the blood-testis barrier, compromising its integrity and leading to impaired spermatogenesis and male infertility. Phthalates, particularly di(2-ethylhexyl) phthalate (DEHP), are widely used plasticizers known to induce BTB disruption through oxidative stress and downregulation of tight junction proteins such as the claudins, namely (*CLDN11*) and occludin (*OCLN*), resulting in germ cell loss and impaired Sertoli cell function[201]. Similarly, bisphenol A (BPA), a common endocrine-disrupting compound found in plastics, has been shown to alter the expression of key junctional proteins and activate inflammatory pathways in Sertoli cells, leading to BTB disassembly[202]. Heavy metals like cadmium are also potent BTB toxicants; cadmium exposure disrupts the cytoskeletal structure of Sertoli cells, increases reactive oxygen species (ROS), and causes extensive BTB damage through *MAPK* and *ER* stress-related pathways[169,179,180,203].

Certain therapeutic drugs, including chemotherapeutic agents like cyclophosphamide and antibiotics like tetracycline, have also been shown to impair BTB integrity, likely due to their cytotoxic or pro-inflammatory effects on Sertoli cells and the surrounding microenvironment[204]. Moreover, recent studies have shown that non-steroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen and naproxen, can alter BTB-associated gene expression and affect tight junction formation[205,206]. These findings underscore the BTB's sensitivity to a wide range of xenobiotic exposures and highlight the need for greater regulatory oversight and targeted research to assess male reproductive risks from environmental and pharmaceutical chemicals.

3.1.3 Endogenous Opioid Signaling in the Testis

With the recent recognition of endogenous opioids and their receptors present within the male reproductive tract, exploration and understanding the involvement of the endogenous opioid system and its importance on male reproductive system is warranted. Opioid peptides such as β-endorphins, enkephalins, and dynorphins, along with their receptors—mu (MOR), delta (DOR), and kappa (KOR)—are expressed in various components of the testis, including Sertoli cells[135] While the activation of these receptors has been implied to modulates intracellular signaling pathways involved in the maintenance of hormone production and homeostasis, their ties to Sertoli cells remain mostly unknown. Opioids, particularly those produced by Leydig cells, exert inhibitory effects on Sertoli cells, which are the main structural components of the BTB. Studies have shown chronic opioid treatment can inhibit androgen-binding protein production by Sertoli cells, a process prevented by opioid antagonists like naloxone. This suggests that opioids can influence Sertoli cell function, potentially impacting the integrity of the BTB[123]. In essence, while opioids are not known to directly compromise the physical structure of the blood-testis barrier in a straightforward manner, they can influence the surrounding cells and hormonal environment in ways that might impact BTB function and the protective mechanisms it provides to developing germ cells. Opioid-mediated signals have been speculated to play a role in the cyclic restructuring of the BTB, a process vital for allowing preleptotene spermatocytes to cross into the adluminal compartment during spermatogenesis[133,207]. Studies have shown that endogenous opioid receptor activity influences the secretion of key Sertoli-derived hormones such as inhibin B and androgen-binding protein (ABP), which play essential roles in feedback regulation of the hypothalamic-pituitary-gonadal (HPG) axis and in maintaining local androgen availability[208]. In this context, endogenous opioids act as local modulators, fine-tuning both the endocrine and potentially barrier function via Sertoli cells under physiological conditions. Yet, little evidence exists as to what other impacts the endogenous opioid system may have, and how this modulation may impact the BTB. Further research is needed to fully understand the intricate relationship between opioid signaling and the BTB, especially regarding direct opioid exposure.

3.1.4 Exogenous Opioid Exposure

In contrast to the physiological actions of endogenous opioids, chronic exposure to exogenous opioids, such as morphine, codeine, and fentanyl, has been consistently associated with disruption of the endogenous opioid system, and thus BTB function. These potent synthetic or semi-synthetic opioids cross the BTB and bind with high affinity to the same receptors, resulting in prolonged activation and desensitization, thus leading to disruption. It has been shown that opiates act as paracrine and regulators of Sertoli and Leydig cells in seminiferous tubules and may exert a suppressive effect on Sertoli cell growth and modulate testosterone secretion by Leydig cells[209]. Furthermore, it has been reported that opioids directly reduce testosterone, testicular interstitial fluid and libido, mainly through testicular damage [210]. Thus, opioid use/abuse may also lead to the suppression and dysfunction of the reproductive axis, which can be a possible cause of male infertility [103]. Opioid abuse may result in reduced gonadotropin-releasing hormone (GnRH) from the hypothalamus, luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the pituitary and also gonadal steroids which, in turn, led to hypogonadism[37,102-104,107,108,124], a well-known clinical problem. Therefore, male infertility can occur through testicular dysfunction in germ cells and somatic cells including Sertoli cells, which may be mediated through chemical exposure, including chronic exposure to morphine. The growing amount of evidence showed that chronic morphine decreases semen quality, sperm counts, erectile capacity, and increases sperm DNA fragmentation[211-213]. Morphine exerts deleterious effects on spermatogenesis through either direct suppression of testicular or indirect impairment in the hypothalamic-pituitary-testicular axis[214,215]. Recent studies have suggested that chronic morphine can induce destructive effects by a variety of mechanisms including, increase in reactive oxygen species and oxidative stress[216,217], serum nitric oxide levels[218], apoptosis[219,220], the levels of pro-inflammatory mediators such as interleukin IL-1 α, IL-6, IL-12, tumor necrosis factor (TNF-α)[221]and suppression of cellular immunity[222]. Moreover, at the cellular level, chronic opioid exposure has been shown to disrupt Sertoli cell mitochondrial function, elevates oxidative stress, and increases apoptotic signaling, each of which compromises the Sertoli cell's structural and supportive functions[180]. These effects are amplified by opioid-induced reductions in FSH responsiveness and decreased inhibin B secretion, effectively impairing both local and systemic regulation of the reproductive axis[103]. In rodent models, chronic fentanyl administration has been linked to reduced testis weight, diminished sperm output, and altered testicular histology, all consistent with BTB dysfunction[223].

Together, the current body of evidence highlights a critical distinction between the homeostatic role of endogenous opioid signaling in Sertoli cells and the disruptive effects of chronic exposure to exogenous opioids. While the endogenous opioid system contributes to the dynamic regulation of the blood-testis barrier, exogenous opioids such as fentanyl can interfere with these regulatory mechanisms, leading to structural and functional disruption. The BTB is not only a physical barrier but also a highly responsive signaling interface, integrating hormonal, metabolic, and environmental cues essential for maintaining male reproductive function. Understanding how both endogenous and exogenous opioids interact with this barrier is key to uncovering the mechanisms underlying opioid-induced infertility as it is an increasingly relevant concern given the widespread use of opioids among reproductive-aged men. This concept carries important implications for public health, fertility preservation, and assessing the use of opioids in a medical setting.

3.2 PURPOSE OF THE STUDY

The purpose of this study was to determine the effects of opioid use directly on human Sertoli cells and thus the blood-testis barrier *in vitro*. In the wake of the ongoing opioid epidemic within the United States, evidence suggests that fentanyl is now the primary driver of abuse and overdose among reproductive-aged men. Existing literature suggests that exogenous opioids have potentially harmful effects on the male reproductive system, particularly by disturbing hormone regulation and disrupting the HPG axis, leading to hypogonadism. In addition, studies have observed specific effects in the testis, particularly in Sertoli cells, along with alterations in sperm health and decreased semen parameters. These studies suggest opioids can have drastic effects on the male reproductive system through acting upon the endogenous opioid system via systemic exposure. However, there is little knowledge on the direct effect exogenous opioids have on the critical cells required for proper human male reproductive success, and whether alterations in these cells and their functional barrier contribute to the negative outcomes seen with opioid use. Specifically, there is little research investigating the direct effect of exogenous opioids on critical cells and functions of male reproduction, such as the Sertoli cells and the BTB. Moreover, specific mechanisms in which opioids act on these aspects in the male reproductive tract remain undetermined.

3.3 STUDY INNOVATION

To address this knowledge gap, the present study investigates the direct effects of fentanyl, a potent synthetic opioid, on Sertoli cells and the human blood-testis barrier. Due to the inherent limitations in studying human reproductive toxicology *in vivo*, we employed a novel *in vitro* model of the human BTB to examine how fentanyl may compromise the health, function, structure, and integrity of this critical testicular barrier. Our approach integrates multiple complementary methodologies, including a physiologically relevant human BTB model, barrier integrity and permeability assays, cellular toxicity assays to assess Sertoli cell viability and health, as well as the expression analysis of Sertoli cell and BTB-relevant genes. Through this comprehensive experimental design, we aim to elucidate the mechanisms by which opioids impair BTB function and contribute to male reproductive dysfunction.

To observe alterations in BTB function and structure, we utilized our in vitro human BTB model paired with both Transepithelial Electrical Resistance (TEER) assays and Paracellular Flux Assays (dye flux) in order to assess phenotypical changes in barrier dynamics. Together, these assays allow for the detection in changes in barrier permeability and electrical resistance; both of which are indicators of an impacted barrier, and thus BTB health and viability.

To investigate the potential cellular mechanisms underlying blood-testis barrier disruption in response to opioid exposure, we assessed markers of apoptosis and oxidative stress in human Sertoli cells exposed to fentanyl. Sertoli cells are essential for maintaining testicular immune privilege and supporting germ cell development, and their viability is critical for BTB integrity. Because opioids have been implicated in testicular dysfunction, we sought to determine whether fentanyl-induced cytotoxicity could contribute to BTB compromise via increased programmed cell death. Annexin V assays were used to quantify early and late apoptotic events across a range of clinically relevant fentanyl concentrations in our *in vitro* model.

In parallel, we evaluated oxidative stress as a potential mediator of Sertoli cell dysfunction, given prior evidence that opioids can disrupt redox homeostasis in peripheral tissues. Oxidative stress is known to perturb tight junction dynamics and activate stress signaling pathways, both of which may undermine BTB structure and function. Assessing reactive oxygen species (ROS) production in our model enabled us to distinguish whether BTB vulnerability was associated with apoptotic loss of Sertoli cells or with sub-lethal but functionally significant oxidative damage.

In conjunction with the previously mentioned assays, we utilized RT-qPCR to examine the expression changes of specific genes related to Sertoli cell and BTB function, health, oxidative stress, DNA repair, and opioid signaling. In total, these endpoints allow for the robust observation of alterations due to opioid use, as well as a potential mechanistic framework to understand how exogenous opioid use may directly compromise male reproductive health at the cellular level.

3.4 RESULTS: OPIOID USE SIGNIFICANTLY ALTERS BLOOD-TESTIS BARRIER INTEGRITY AND PERMEABILITY

This study aimed to determine the effects of opioid use directly on human Sertoli cells and thus the blood-testis barrier *in vitro*. Existing literature suggests that opioids have potentially harmful effects on the male reproductive system, particularly by disturbing hormone regulation and disrupting the HPG axis, leading to hypogonadism. In addition, studies have observed alterations in sperm health in opioid users, noting decreased semen parameters. These studies suggest opioids can have drastic effects on the male reproductive system, as both endogenous opioids and their respective receptors are present within this specialized niche, or, the seminiferous tubules, and suspected to be functionally important. Previous studies in animal models found that opioid use has been linked to reduced testis weight, diminished sperm output, and altered testicular histology, all of which point towards a disruption of the blood-testis barrier[180,223]. However, there is little knowledge on the direct effect exogenous opioids have on the critical Sertoli cells and thus the BTB required for proper human male reproductive success, and whether alterations in these cells and their functional barrier contribute to the negative outcomes observed in real-world human populations. Given the importance of the BTB and Sertoli cells in male fertility and reproductive health, we wanted to observe the direct impact of opioids in our *in vitro* human blood-testis barrier model.

To determine the direct effect of opioids on the blood-testis barrier, we cultured human Sertoli cells derived from testis culture along with TRT treated Sertoli cells on Transwell semi-permeable dishes and measured their transepithelial electrical resistance (TEER) to observe their instantaneous transcellular permeability to ions over the given timeframe, alongside measuring the paracellular flux of various sized solutes after the dosing duration. Cultured human Sertoli cells were plated on Transwell membranes at optimal confluency to allow for proper apical and basal barrier formation as well as cytoskeletal and junction formation to mimic a human *in vivo* BTB. an Epithelial

Voltohmmeter was used to determine healthy cell-barrier formation via probing the medium on both sides of the Transwell dish. After allowing the cells to acclimate, the human Sertoli cells were treated with clinically relevant doses fentanyl at 0 ng (control), 2 ng, 20 ng, or 200 ng, based off serum level concentrations in men after prescribed medical use. TEER measurements were recorded alongside medium changes occurring every other day for 10 days. Both human primary Sertoli cells (Figure 3.1 A-B) and human TRT Sertoli cells (Figure 3.2 A-B) were used to determine the impacts of exogenous opioids on the BTB. Human TRT cells were used for the remainder of the following experiments as they were found to be comparable to the primary cells in both expression and BTB formation but were far more stable in culture for longer durations which were required to conduct our analyses. Furthermore, an additional TEER analysis (Figure 3.3) was conducted over an extended period following opioid cessation to determine the impact on barrier resistance measurements.

To conduct the Paracellular Flux assay, TEER was measured on the 10th day, the fentanyl-treated human Sertoli cells were equilibrated with a specialized medium, then the apical chamber was replaced with fluorescent-labeled dextran (10 kDa) and calcein (0.63 kDa) as described. Over the course of two hours, media was collected from the basal side of the chamber in 30 minute intervals, and prepared in triplicate on a 96-well plate for measuring the intensity on a microplate reader to determine the differences in the amount of flux between calcein or dextran appearing in the lower chamber of the Transwell over time for each dosing group (Figure 3.4 A-B).

In summary, both human primary Sertoli cells (Figure 3.1 A-B) and human TRT Sertoli cells (Figure 3.2 A-B) showed similar trends in TEER measurements (graphs feature combined plots as well as separated plots by dose for visibility), with notable significant increases in barrier resistance. The human Sertoli cell cessation TEER results (Figure 3.3) revealed a similar trend in the initial 10-day dosing duration as well as significantly elevated barrier resistance 5 days post abstinence from fentanyl dosing alongside regular medium changes. Paracellular flux assays of various sized solutes (Figure 3.4 A-B) revealed significant changes at high doses of fentanyl for small molecules only, with the other doses having no significance, as well as no significant changes in the flux of large molecules.

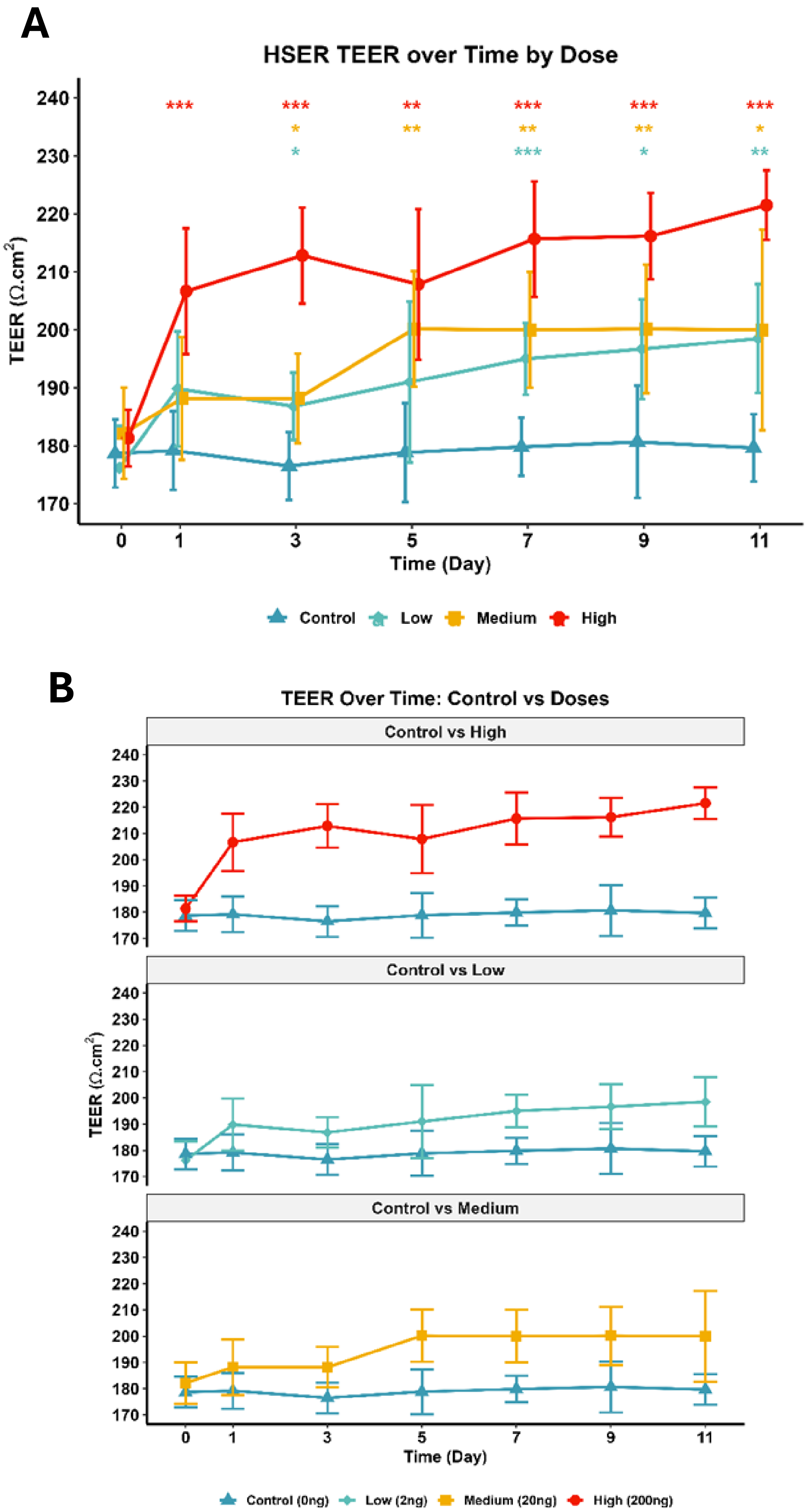

A
HSER TEER over Time by Dose
TEER (Ω.cm²)
240
230
220
210
200
190
180
170
***
***
*
*
**
**
***
**
***
***
**
*
***
*
**
0
1
3
5
7
9
11
Time (Day)
Control
Low
Medium
High
B
TEER Over Time: Control vs Doses
Control vs High
Control vs Low
Control vs Medium
TEER (Ω.cm²)
240
230
220
210
200
190
180
170
0
1
3
5
7
9
11
Time (Day)
Control (0ng)
Low (2ng)
Medium (20ng)
High (200ng)

Figure 3.1. Long-term opioid use alters the Transepithelial Electrical Resistance (TEER) of human Sertoli cell BTB model *in vitro*. To evaluate the impact of fentanyl exposure on the human blood-testis barrier (BTB), transepithelial electrical resistance (TEER) was measured in human Sertoli cells over an 11-day treatment period. (A) Represents combined graph, (B) represents separated by dose compared to control. Cells were exposed every other day to 0 ng (control), 2 ng (low), 20 ng (medium), or 200 ng (high) fentanyl, and TEER ($\Omega \cdot cm^2$) values were recorded at 0, 1, 3, 5, 7, 9, and 11 days. TEER measurements were conducted in quadruplicate per condition ($n = 4$). A two-way ANOVA with Dunnett's multiple comparisons test was used to determine statistical significance compared to the control group at each time point. Data are shown as mean ± SEM. Significance is indicated as: $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

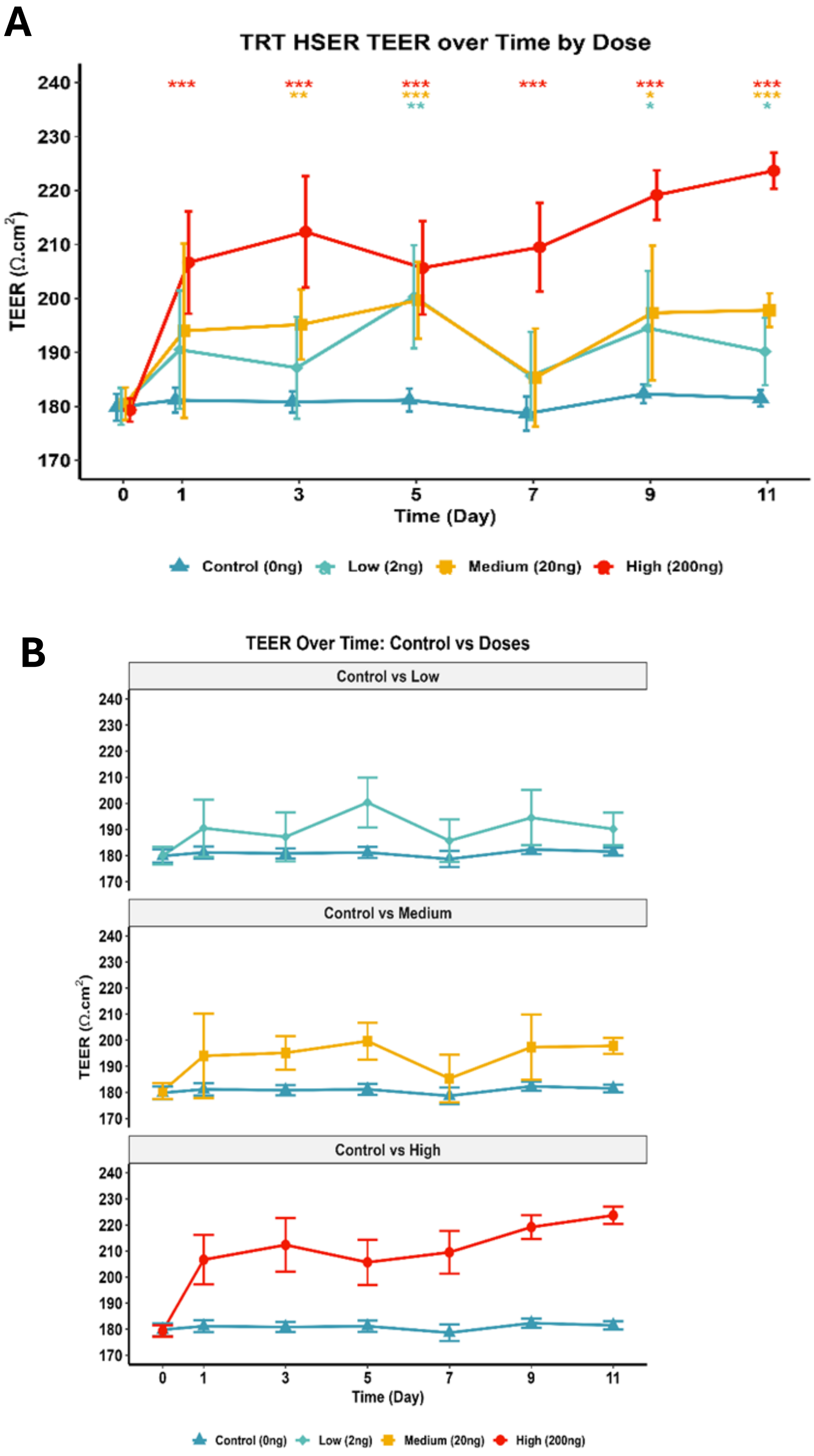
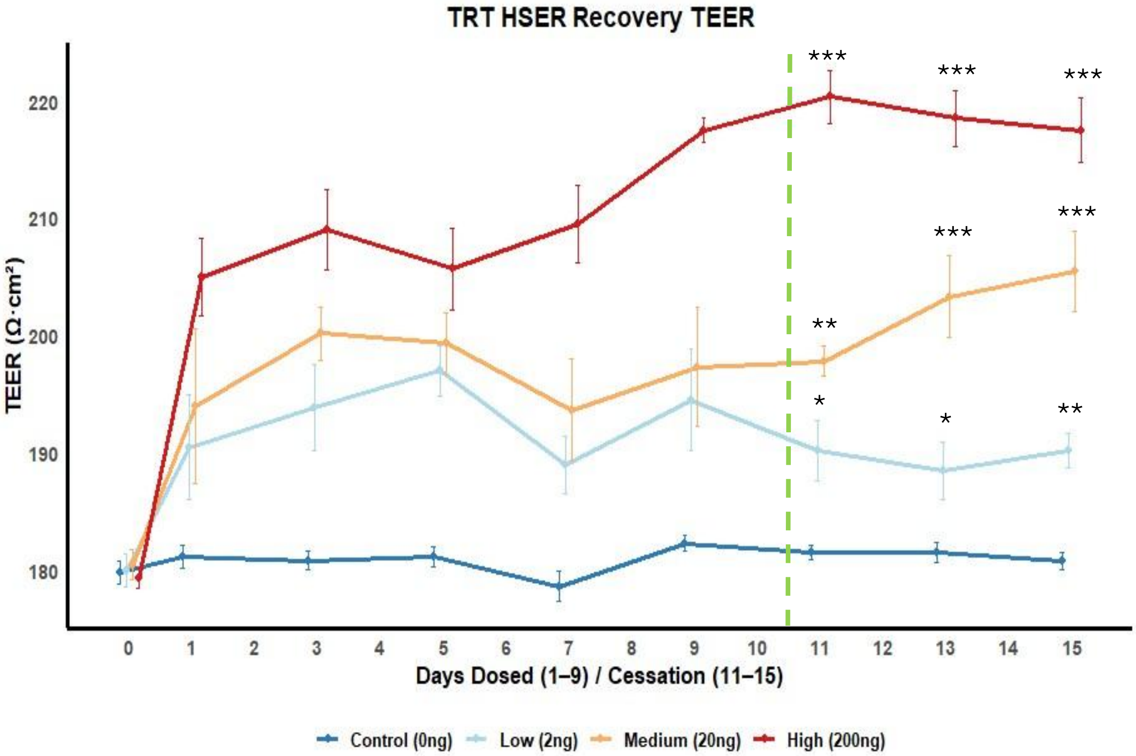

A
TRT HSER TEER over Time by Dose
TEER (Ω.cm²)
240
230
220
210
200
190
180
170
***
***
**
***
***
**
***
***
*
*
***
***
*
0
1
3
5
7
9
11
Time (Day)
Control (0ng)
Low (2ng)
Medium (20ng)
High (200ng)
B
TEER Over Time: Control vs Doses
Control vs Low
Control vs Medium
Control vs High
TEER (Ω.cm²)
240
230
220
210
200
190
180
170
0
1
3
5
7
9
11
Time (Day)
Control (0ng)
Low (2ng)
Medium (20ng)
High (200ng)

Figure 3.2. Long-term opioid use alters the Transepithelial Electrical Resistance (TEER) of human TRT Sertoli cell BTB model *in vitro*. To evaluate the impact of fentanyl exposure on blood-testis barrier (BTB), transepithelial electrical resistance (TEER) was measured in human TRT Sertoli cells over an 11-day treatment period. Cells were exposed daily to 0 ng/mL (control), 2 ng/mL (low), 20 ng/mL (medium), or 200 ng/mL (high) fentanyl, and TEER ($\Omega \cdot cm^2$) values were recorded at 0, 1, 3, 5, 7, 9, and 11 days. (A) Represents combined graph, (B) represents separated by dose compared to control. TEER measurements were conducted in quadruplicate per condition (n=4). A one-way ANOVA with Dunnett's multiple comparisons test was used to determine statistical significance compared to the control group at each time point. Data are shown as mean ± SEM. Significance is indicated as: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.



TRT HSER Recovery TEER
TEER (Ω·cm²)
220
210
200
190
180
Days Dosed (1–9) / Cessation (11–15)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
***
***
***
***
***
**
*
*
**
Control (0ng)
Low (2ng)
Medium (20ng)
High (200ng)

Figure 3.3. Elevated Transepithelial Electrical Resistance (TEER) of *in vitro* human Sertoli cell BTB model after cessation from long-term opioid use. Transepithelial electrical resistance (TEER) was measured in an *in vitro* human Sertoli cell model of the blood-testis barrier (BTB) across a 15-day experimental timeline, consisting of 10 days of fentanyl exposure and a 5-day withdrawal phase. Cells were treated with fentanyl at Control (0 ng/mL), Low (2 ng/mL), Medium (20 ng/mL), or High (200 ng/mL) doses. TEER was recorded every other day to monitor dynamic changes in barrier function. Each data point represents the mean ± SEM from three independent replicates (n=3). A two-way ANOVA was performed to assess the interaction between time and dose, followed by Dunnett's multiple comparisons test to identify significant differences from the control group at each timepoint. Asterisks denote significance where *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

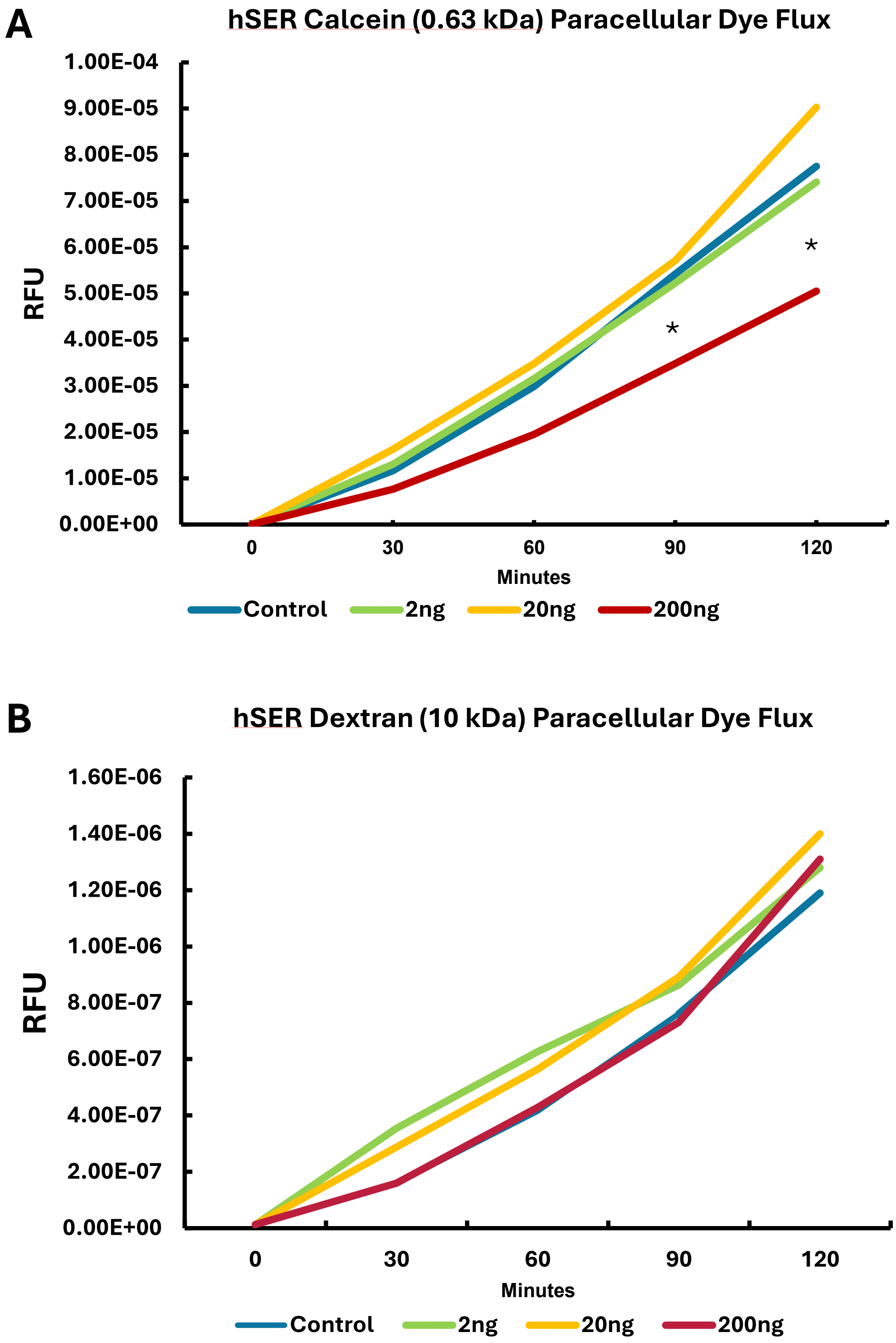
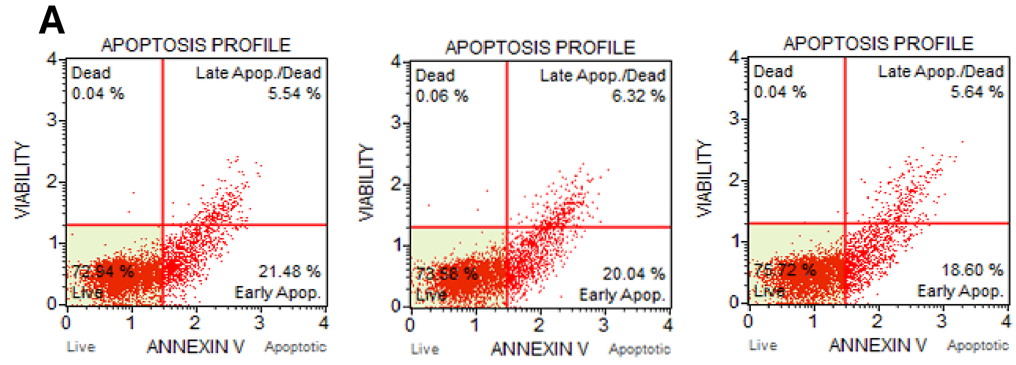

A
hSER Calcein (0.63 kDa) Paracellular Dye Flux
RFU
1.00E-04
9.00E-05
8.00E-05
7.00E-05
6.00E-05
5.00E-05
4.00E-05
3.00E-05
2.00E-05
1.00E-05
0.00E+00
*
*
0
30
60
90
120
Minutes
Control
2ng
20ng
200ng
B
hSER Dextran (10 kDa) Paracellular Dye Flux
RFU
1.60E-06
1.40E-06
1.20E-06
1.00E-06
8.00E-07
6.00E-07
4.00E-07
2.00E-07
0.00E+00
0
30
60
90
120
Minutes
Control
2ng
20ng
200ng

Figure 3.4. Long-term opioid use altered small, but not large molecule permeability of human Sertoli cell BTB model *in vitro*. To measure molecular permeability in the blood-testis barrier, dye flux assays were conducted over a period of 2 hours with measurements occurring every 30 minutes (0 – 120min). Figure (A) represents the dye flux change over time in small molecule permeability (calcein 0.63 kDa), while (B) represents large molecule permeability (dextran 10 kDa). Each data point represents the mean ± SEM from six independent replicates (n=6) for each dosing group. Significant alterations in dye flux permeability were determined by ANOVA with Dunnett's multiple comparisons test, where ns is not significant, * is $p < 0.05$, ** is $p < 0.01$, and *** is $p < 0.001$.

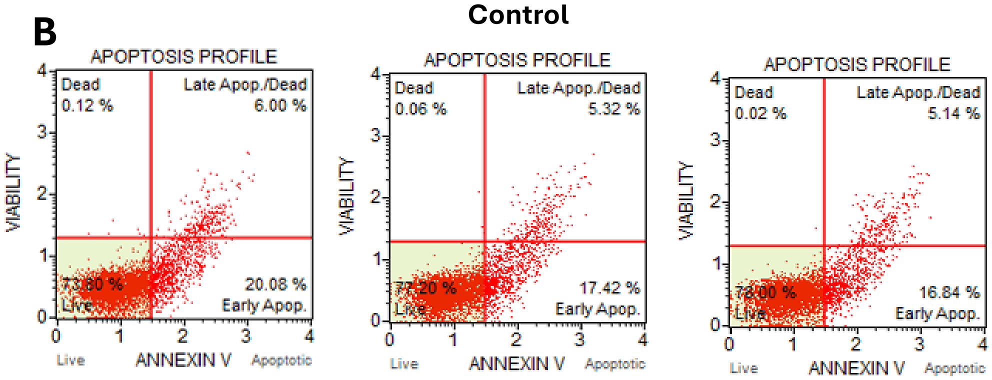
3.4 RESULTS: OPIOID USE DID NOT SIGNIFICANTLY ALTER SERTOLI CELL VIABILITY

Several environmental toxicants have been shown to decrease the viability of spermatogenic cells as well as the BTB[13,14,140,164,224]. Opioids have been shown to induce apoptosis in other cell types after exposure[223,225], however information on opioid exposure on human Sertoli cells and the BTB remains limited. Few studies have shown alterations in Sertoli cell health and viability in response to opioid exposure[134,180]. While these studies provide relevant insight, there are none examining these opioid driven effects directly, or, moreover, without the involvement of the impacts on the nervous and endocrine systems, both known modulators in the male reproductive system. Moreover, most studies involved the use of animal models, warranting cross examination in humans. Given the importance of both Sertoli cells and the BTB in male reproductive health and function, it is crucial to investigate what direct impacts opioid use may have on cell health and viability in this specialized niche.

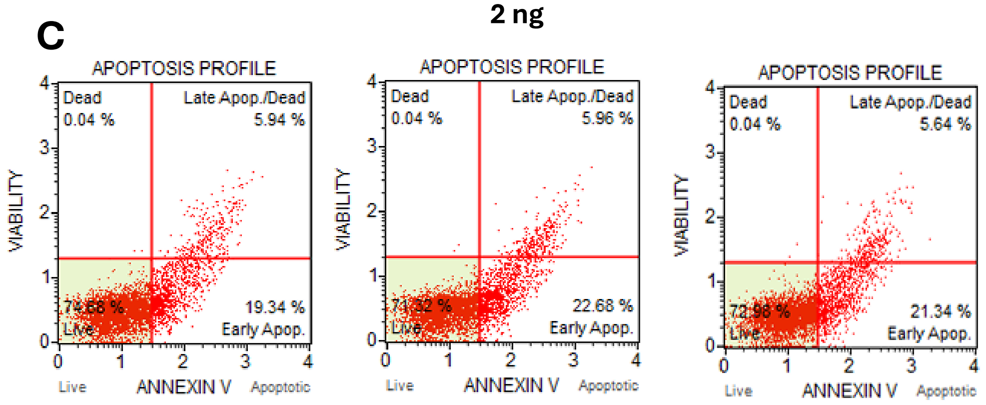
Here, we examined whether clinically relevant human male serum level doses of fentanyl affect cell viability using our *in vitro* human BTB model. Sertoli cells are essential for maintaining testicular immune privilege and supporting germ cell development, and their viability is critical for BTB integrity. Because opioids have been implicated in testicular dysfunction, we sought to determine whether fentanyl-induced cytotoxicity could contribute to BTB compromise via increased programmed cell death. As mentioned, few but noteworthy research had observed increases in total apoptotic cell populations after opioid exposure in animal models[180]. To determine if this same mechanism was driving the observed disruptions in BTB permeability and electrical resistance, we cultured human Sertoli cells on Transwell semi-permeable dishes at a biologically relevant barrier density allowing for proper apical and basal arrangement and barrier formation alongside cytoskeletal and junction formation, as previously described for our TEER experiments. As before, human Sertoli cells were treated with clinically relevant doses fentanyl at 0 ng/mL (control), 2 ng/mL, 20 ng/mL, or 200 ng/mL, to simulate serum concentration levels of opioids in men. After barrier establishment a 10-day dosing period occurring alongside medium changes took place every other day. On the 10th day after TEER measurements, we assessed cell viability by using Annexin V to detect apoptotic cells to indicate cell health via the Muse® Annexin V & Dead Cell Kit. Flow cytometry analyses allowed for the assessment of live, early apoptotic, late apoptotic, and dead cell population percentages in our *in vitro* BTB model Sertoli cells treated with our dosing range of fentanyl, respectively (Figure 3.5 A-D). Alterations in cell populations and thus viability were compared against our control dose and represented graphically (Figure 3.6 A-D). Our results showed that fentanyl did not significantly alter human Sertoli cell viability across the dosing groups compared to the control.



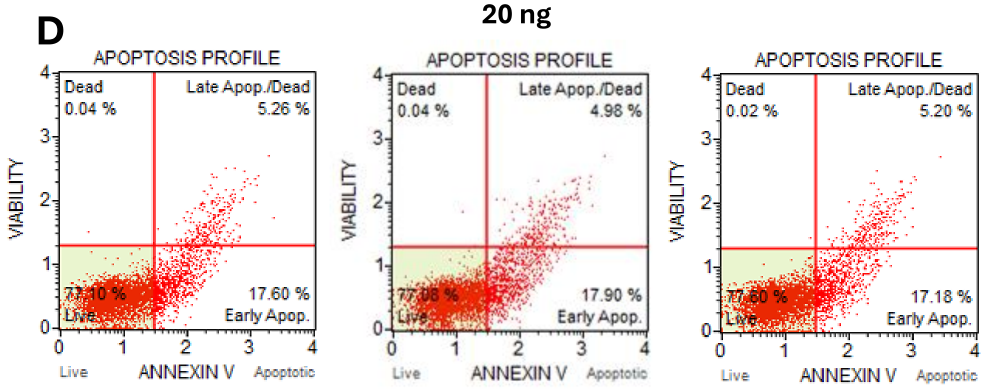
Control



2 ng

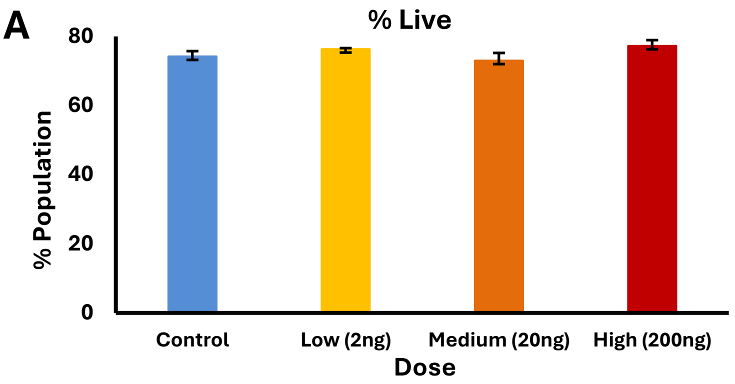


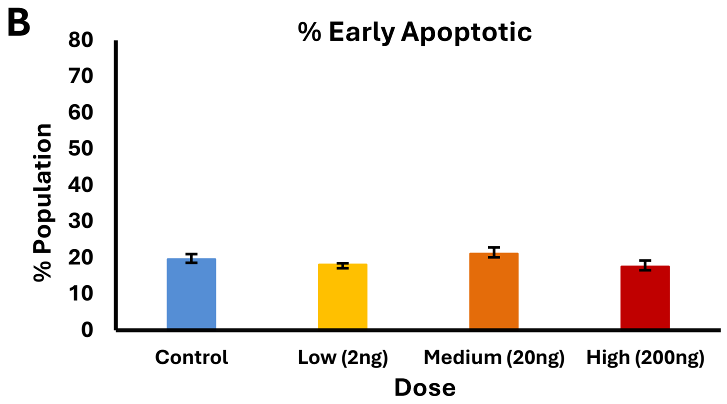
20 ng

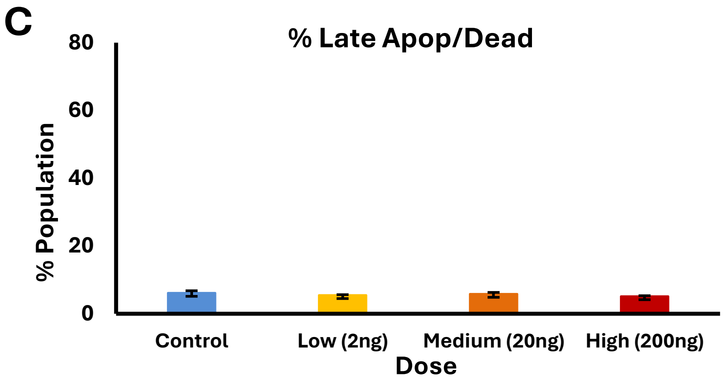


200 ng

Figure 3.5. Annexin V Flow Cytometry Assay populations of opioid-exposed human Sertoli cells. To assess the effect of escalating fentanyl doses on cell viability and apoptotic states, human Sertoli cells were exposed to 0 ng/mL (control), 2 ng/mL, 20 ng/mL, and 200 ng/mL fentanyl and analyzed by Annexin V/PI staining. The percentage of cells in each state—(A) Live, (B) Early Apoptotic, (C) Late Apoptotic, and (D) Total Apoptotic—was quantified by flow cytometry. Each condition was performed in three biological replicates ($n = 3$). Significant differences in population percentages across doses were evaluated using one-way ANOVA followed by Tukey's multiple comparisons test. Data are presented as mean ± standard error of the mean (SEM). No statistically significant differences were observed between groups in any apoptotic stage ($p > 0.05$).


A
% Live
% Population
0
20
40
60
80
Control
Low (2ng)
Medium (20ng)
High (200ng)
Dose


B
% Early Apoptotic
% Population
0
10
20
30
40
50
60
70
80
Control
Low (2ng)
Medium (20ng)
High (200ng)
Dose


C
% Late Apop/Dead
% Population
0
20
40
60
80
Control
Low (2ng)
Medium (20ng)
High (200ng)
Dose

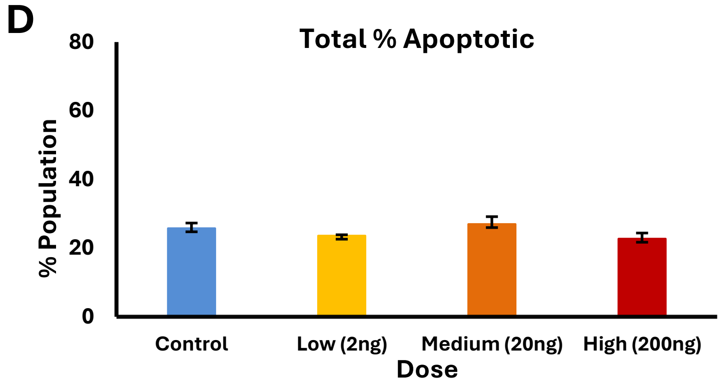

D
Total % Apoptotic
% Population
0
20
40
60
80
Control
Low (2ng)
Medium (20ng)
High (200ng)
Dose

Figure 3.6. Fentanyl did not significantly alter apoptotic populations or cell viability in human Sertoli cells *in vitro*. To assess the effect of escalating fentanyl doses on cell viability and apoptotic states, human Sertoli cells were exposed to 0 ng/mL (control), 2 ng/mL, 20 ng/mL, and 200 ng/mL fentanyl and analyzed by Annexin V/PI staining. The percentage of cells in each state—(A) Live, (B) Early Apoptotic, (C) Late Apoptotic, and (D) Total Apoptotic—was quantified by flow cytometry. Each condition was performed in three biological replicates ($n = 3$). Significant differences in population percentages across doses were evaluated using one-way ANOVA followed by Tukey's multiple comparisons test. Data are presented as mean ± standard error of the mean (SEM). No statistically significant differences were observed between groups in any apoptotic stage ($p > 0.05$).

3.4 RESULTS: OPIOID USE SIGNIFICANTLY INDUCED ROS AND INCREASED OXIDATIVE STRESS IN SERTOLI CELLS

Several environmental toxicants have been shown to increase reactive oxygen species and thus oxidative stress across a wide array of cell types, including those within the male reproductive tract[13,14,140,164,224]. Numerous studies have implicated the role of oxidative stress and reactive oxygen species (ROS) in cell health and disease, however information on these effects in human Sertoli cells and the implications for the blood-testis barrier (BTB) and thus male reproductive health is rather limited. Sertoli cells are essential for maintaining testicular immune privilege and supporting germ cell development. Oxidative stress is known to perturb tight junction dynamics and activate stress signaling pathways, both of which may undermine BTB structure and function. A recent study observed an increase in ROS after morphine exposure in rodents[180], however it is unknown whether these conditions are present in human Sertoli cells, as well as if it is due to a direct impact of opioids on the Sertoli cells and not a systemic effect governed by the HPG axis. Knowing that opioids have been implicated in testicular dysfunction, we evaluated oxidative stress as a potential mediator of Sertoli cell dysfunction, given prior evidence that opioids can disrupt redox homeostasis in peripheral tissues.

Given that our transepithelial electrical resistance (TEER) and paracellular dye flux assays revealed significant alterations in barrier resistance and permeability, while Sertoli cell populations remained statistically healthy in our apoptosis assay, we sought to determine whether ROS and thus oxidative stress could be mechanistically driving the observed phenotypes. Assessing ROS production in our *in vitro* human BTB model enabled us to distinguish whether BTB vulnerability and the observed barrier changes were associated with functionally significant oxidative damage and construct a mechanistic framework to understand how opioids may compromise male reproductive health and the BTB at the cellular level.

To investigate the potential cellular mechanisms underlying blood-testis barrier disruption in response to opioid exposure, we assessed markers of ROS and oxidative stress in human Sertoli cells in our *in vitro* BTB model after chronic exposure to the opioid fentanyl at clinically relevant doses as previously described. Briefly, we cultured human Sertoli cells on Transwell semi-permeable dishes. Cells were cultured and grown to a density for proper apical and basal barrier formation alongside cytoskeletal and junction formation to model *in vivo* barriers. TEER was used to determine healthy cell-barrier formation. After the measurement, the cells were treated every other day with clinically relevant doses fentanyl at 0 ng/mL (control), 2 ng/mL, 20 ng/mL, or 200 ng/mL, as well as a positive control of hydrogen peroxide (H_2O_2) to simulate long-term exposure over the course of 10 days, with dosing occurring every other day alongside medium changes.

On the 10th day, positive control cells were dosed with H_2O_2 for a period of 2 hours following the assay protocol, occurring prior to cell harvest and preparation. Assessments were conducted with a Muse® Oxidative Stress ROS assay. Cytometry plots of the observed ROS measurements (Figure 3.7 A-E) denote population percentages displaying positive or negative ROS metrics. Graphical representations and analyses (Figure 3.8) reveal overall significance in positive ROS(+) cells for each dose in comparison to a negative control (0 ng/mL fentanyl) and positive control (H_2O_2). In summary, we observed significantly increased ROS and thus oxidative stress in human Sertoli cells from our *in vitro* human BTB model, notably in a dose-dependent manner from fentanyl exposure.

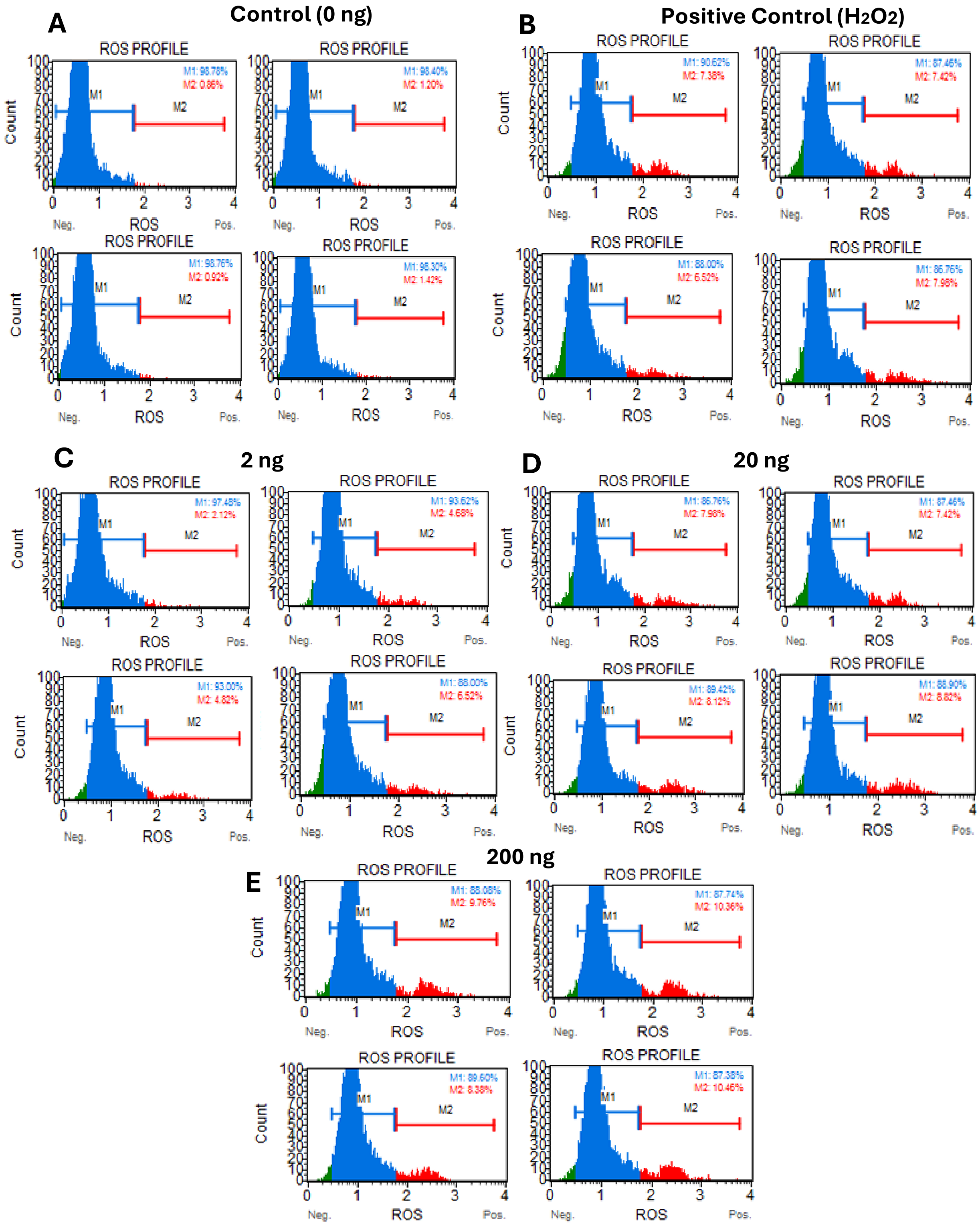

A
Control (0 ng)
ROS PROFILE
M1: 98.78%
M2: 0.86%
ROS PROFILE
M1: 98.40%
M2: 1.20%
ROS PROFILE
M1: 98.76%
M2: 0.92%
ROS PROFILE
M1: 98.30%
M2: 1.42%
B
Positive Control (H2O2)
ROS PROFILE
M1: 90.62%
M2: 7.38%
ROS PROFILE
M1: 87.46%
M2: 7.42%
ROS PROFILE
M1: 88.00%
M2: 6.52%
ROS PROFILE
M1: 86.76%
M2: 7.98%
C
2 ng
ROS PROFILE
M1: 97.48%
M2: 2.12%
ROS PROFILE
M1: 93.62%
M2: 4.68%
ROS PROFILE
M1: 93.00%
M2: 4.82%
ROS PROFILE
M1: 88.00%
M2: 6.52%
D
20 ng
ROS PROFILE
M1: 86.76%
M2: 7.98%
ROS PROFILE
M1: 87.46%
M2: 7.42%
ROS PROFILE
M1: 89.42%
M2: 8.12%
ROS PROFILE
M1: 88.90%
M2: 8.82%
E
200 ng
ROS PROFILE
M1: 88.08%
M2: 9.76%
ROS PROFILE
M1: 87.74%
M2: 10.36%
ROS PROFILE
M1: 89.60%
M2: 8.38%
ROS PROFILE
M1: 87.38%
M2: 10.46%
M1
M2
Count
0
10
20
30
40
50
60
70
80
90
100
0
1
2
3
4
Neg.
ROS
Pos.

Figure 3.6. ROS Flow Cytometry populations of opioid-exposed human Sertoli cells. To quantify intracellular oxidative stress, human Sertoli cells were treated with 0 ng/mL (control), 2 ng/mL, 20 ng/mL, or 200 ng/mL fentanyl for 10 days. A positive control group was included using a known ROS inducer, hydrogen peroxide (H_2O_2). Reactive oxygen species (ROS)-positive cells were identified via staining and quantified using flow cytometry. Each condition was analyzed across four replicates (n = 4). A one-way ANOVA followed by Tukey's multiple comparisons test was used to assess statistical significance between groups. Data are shown as mean ± standard error of the mean (SEM). Significant increases in %ROS(+) cells were observed across each fentanyl dose. Significance denoted as follows: ns = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

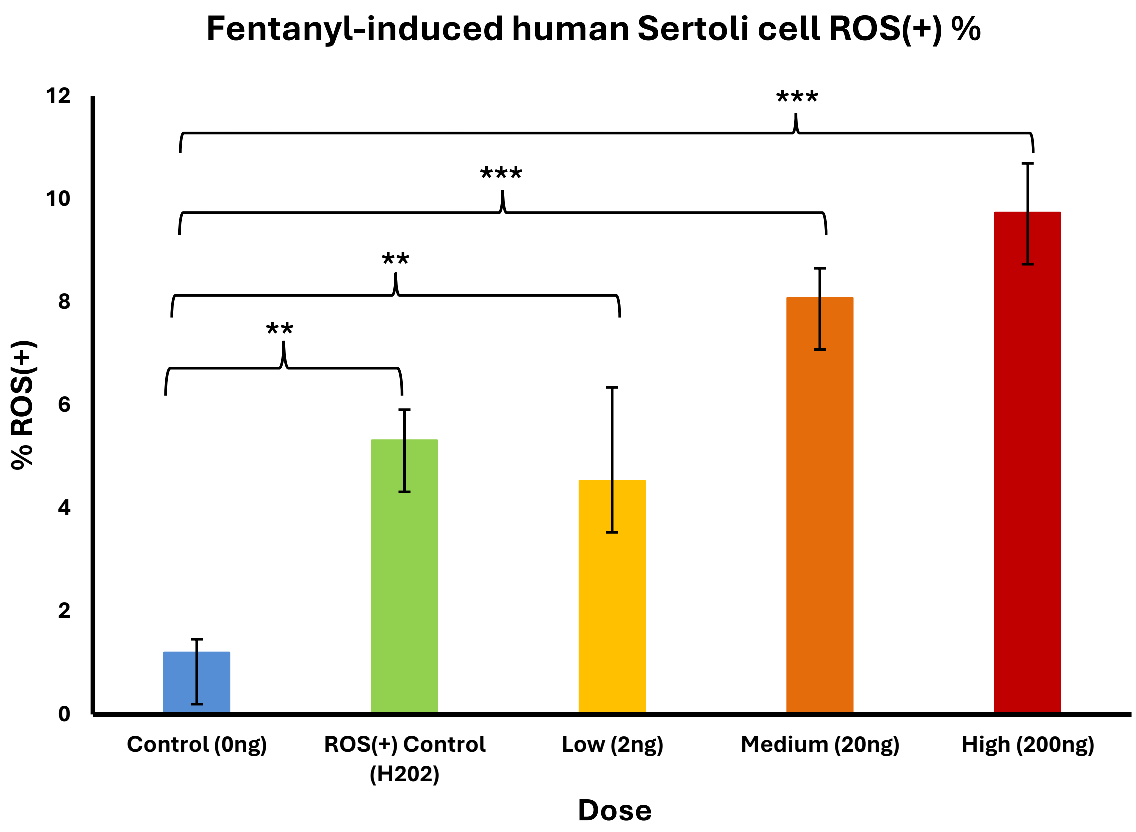

Fentanyl-induced human Sertoli cell ROS(+) %
% ROS(+)
0
2
4
6
8
10
12
**
**
***
***
Control (0ng)
ROS(+) Control (H202)
Low (2ng)
Medium (20ng)
High (200ng)
Dose

Figure 3.8. Fentanyl significantly increases ROS in human Sertoli cells *in vitro*. To quantify intracellular ROS generation and oxidative stress, human Sertoli cells were treated with 0 ng/mL (control), 2 ng/mL, 20 ng/mL, or 200 ng/mL fentanyl for 10 days with dosing occurring every other day. A positive control group was included using a known ROS inducer (H_2O_2). ROS-positive cells were identified via staining and quantified using flow cytometry. Each condition was analyzed across 4 replicates ($n = 4$). A one-way ANOVA followed by Tukey's multiple comparisons test was used to assess statistical significance between groups. Data are shown as mean ± standard error of the mean (SEM). Significant increases in %ROS(+) cells were observed in a dose-dependent manner. Significance denoted as follows: ns = not significant, ** = $p < 0.01$, *** = $p < 0.001$.

3.4 RESULTS: OPIOID-INDUCED ALTERATIONS IN HUMAN SERTOLI CELL GENE EXPRESSION

Given the results from both the current findings in literature as well as the results present thus far in our study, we sought to investigate the molecular impact of opioid exposure on the integrity and function of the human blood-testis barrier (BTB) and Sertoli cells. To do this, we performed a gene expression analysis in human Sertoli cells following our fentanyl treatment as previously described. Briefly, TRT treated Sertoli cells were cultured and subsequently plated on Transwell semi-permeable dishes at a density allowing for proper apical and basal barrier formation alongside cytoskeletal and junction formation. After TEER was used to determine healthy cell-barrier formation pre-dosing, the cells were treated with clinically relevant doses fentanyl at 0 ng/mL (control), 2 ng/mL, 20 ng/mL, or 200 ng/mL, to simulate different serum level concentrations of opioids found in men. Dosing occurred during medium changes every other day for the course of 10 days. On the 10th day, the cells were harvested, and RNA was extracted and quantified for the creation of cDNA for RT-qPCR gene expression analysis.

Primers for RT-qPCR were selected for genes associated with key cellular processes relevant to BTB physiology and Sertoli cell health and function, including junctional integrity, hormone responsiveness, redox regulation, and DNA damage response. *GAPDH* was used as a housekeeping gene for normalization. Overall, 13 gene expression levels were selected to investigate the prior findings from our BTB and Sertoli cell assays as well as explore additional avenues involved in opioid-induced alterations described in the literature.

Tight junction-associated genes *CLDN1*, *CLDN3*, and *CLDN7* were included to assess whether fentanyl alters the expression of proteins central to BTB structure (Figure 3.9 A-C). We investigated the gene expression levels of both *CAT* and *SOD1* which are involved in reactive oxygen species detoxification (Figure 3.10 A-C). *AR, SOX9,* and *GATA4*, were examined as Sertoli cell critical markers and essential regulators of Sertoli cell differentiation and hormonal responsiveness (Figure 3.11 A-C). To explore pathways potentially involved in our observed opioid-induced BTB disruptions, we measured expression of *ATR* and *ATM* (Figure 3.12 A-B), and *CASP9* and *XRCC1* (Figure 3.13 A-B), which are involved in apoptotic signaling pathways, and genomic stability via DNA repair pathways.

Finally, *OPRM1*, encoding the μ-opioid receptor, was assessed to observe receptor-specific effects in our model (Figure 3.14). Together, this targeted gene panel was designed to capture expression and thus potential transcriptional changes in Sertoli cells that may contribute to BTB dysfunction in the context of our observed opioid exposure effects. Significant alterations in gene expression were observed at different doses for nearly every gene assessed.

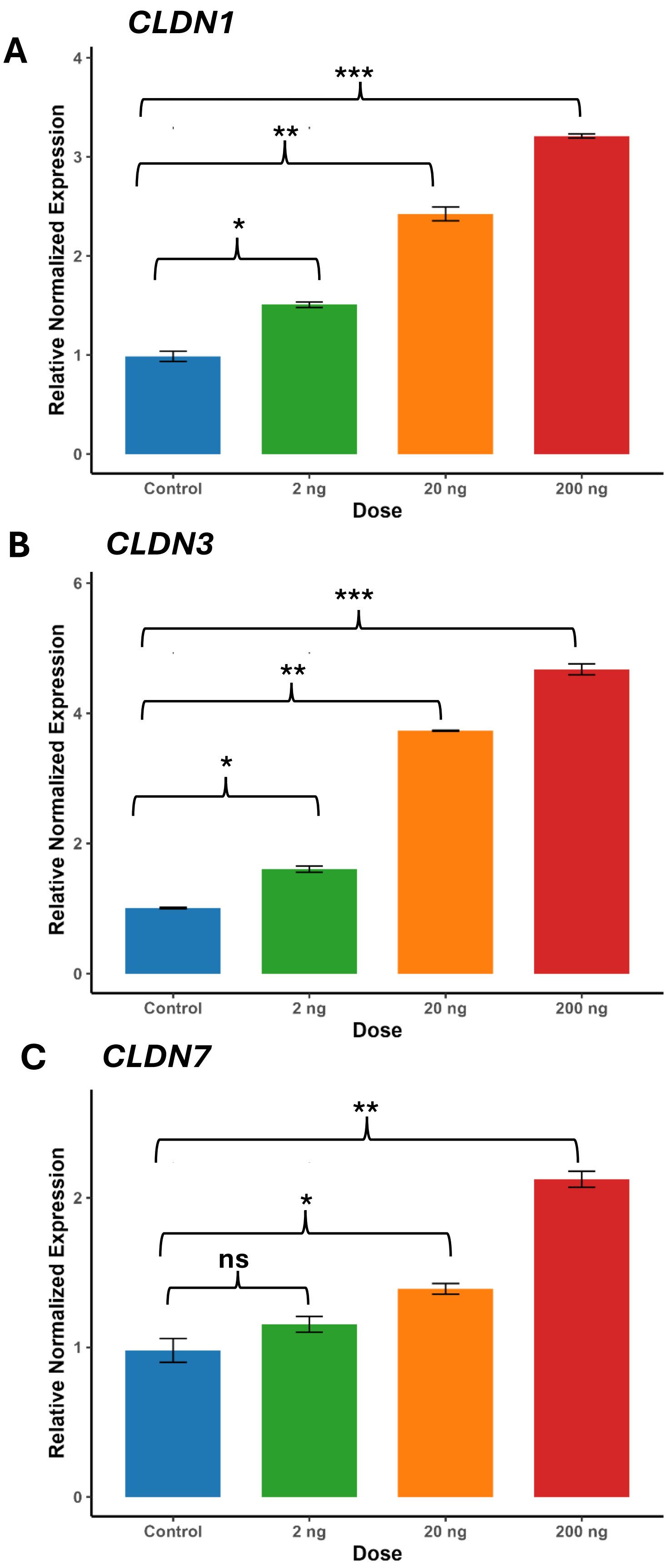

A
CLDN1
Relative Normalized Expression
0
1
2
3
4
*
**
***
Control
2 ng
20 ng
200 ng
Dose
B
CLDN3
Relative Normalized Expression
0
2
4
6
*
**
***
Control
2 ng
20 ng
200 ng
Dose
C
CLDN7
Relative Normalized Expression
0
1
2
ns
*
**
Control
2 ng
20 ng
200 ng
Dose

Figure 3.9. Gene Expression changes post fentanyl exposure in *CLDN1*, *CLDN3*, and *CLDN7* in human Sertoli cells. To assess transcriptional responses of human Sertoli cells to escalating doses of fentanyl (0 ng/mL, 2 ng/mL, 20 ng/mL, 200 ng/mL), relative normalized gene expression was measured for (A) *CLDN1*, (B) *CLDN3*, and (C) *CLDN7*. Each dose group consisted of 6 biological replicates ($n = 6$), and expression values were normalized to control. A Kruskal-Wallis test followed by Dunn's multiple comparisons test with Bonferroni correction was used to determine statistical significance between each fentanyl dose and control. All data are represented as mean ± standard error. ns indicates not significant, * is $p < 0.05$, ** is $p < 0.01$, and *** is $p < 0.001$.

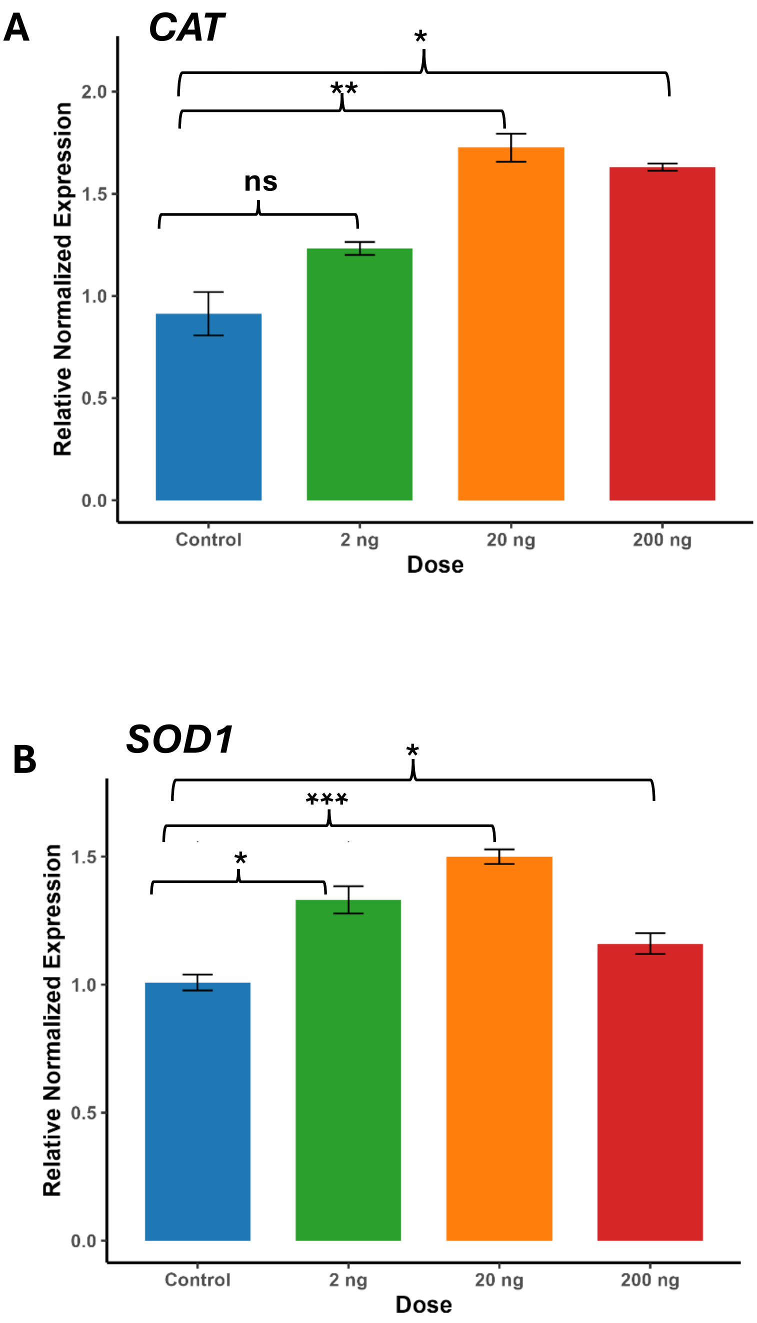

A
CAT
Relative Normalized Expression
0.0
0.5
1.0
1.5
2.0
ns
**
*
Control
2 ng
20 ng
200 ng
Dose
B
SOD1
Relative Normalized Expression
0.0
0.5
1.0
1.5
*
***
*
Control
2 ng
20 ng
200 ng
Dose

Figure 3.10. Gene Expression post fentanyl exposure in *CAT* and *SOD1* in human Sertoli cells. To assess transcriptional responses of human Sertoli cells to escalating doses of fentanyl (0 ng/mL, 2 ng/mL, 20 ng/mL, 200 ng/mL), relative normalized gene expression was measured for: (A) *CAT* and (B) *SOD1*. Each dose group consisted of 6 biological replicates (n = 6), and expression values were normalized to control. A Kruskal-Wallis test followed by Dunn's multiple comparisons test with Bonferroni correction was used to determine statistical significance between each fentanyl dose and control. All data are represented as mean ± standard error. ns indicates not significant, * is $p < 0.05$, ** is $p < 0.01$, and *** is $p < 0.001$.

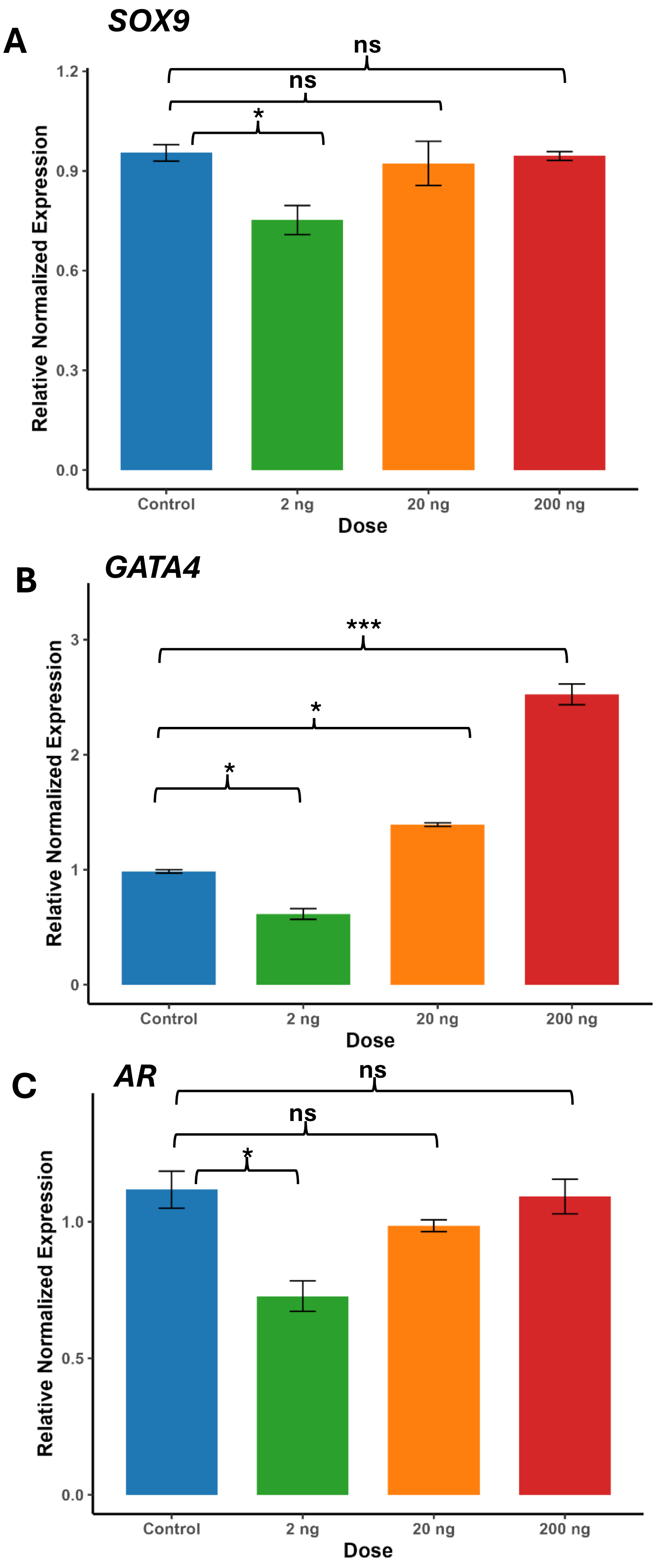
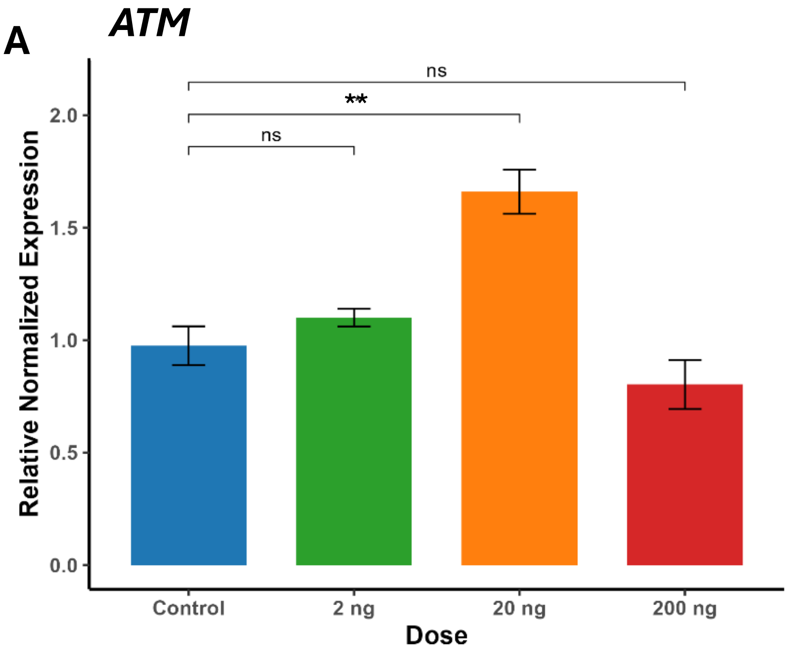

A
SOX9
ns
ns
*
Relative Normalized Expression
1.2
0.9
0.6
0.3
0.0
Control
2 ng
20 ng
200 ng
Dose
B
GATA4
***
*
*
Relative Normalized Expression
3
2
1
0
Control
2 ng
20 ng
200 ng
Dose
C
AR
ns
ns
*
Relative Normalized Expression
1.0
0.5
0.0
Control
2 ng
20 ng
200 ng
Dose

Figure 3.11. Gene Expression changes post fentanyl exposure in *SOX9*, *GATA4*, and *AR* in human Sertoli cells. To assess transcriptional responses of human Sertoli cells to escalating doses of fentanyl (0 ng/mL, 2 ng/mL, 20 ng/mL, 200 ng/mL), relative normalized gene expression was measured for: (A) *SOX9*, (B) *GATA4*, and (C) *AR*. Each dose group consisted of 6 biological replicates ($n = 6$), and expression values were normalized to control. A Kruskal-Wallis test followed by Dunn's multiple comparisons test with Bonferroni correction was used to determine statistical significance between each fentanyl dose and control. All data are represented as mean ± standard error. ns indicates not significant, * is $p < 0.05$, ** is $p < 0.01$, and *** is $p < 0.001$.


A
ATM
ns
**
ns
Relative Normalized Expression
2.0
1.5
1.0
0.5
0.0
Control
2 ng
20 ng
200 ng
Dose

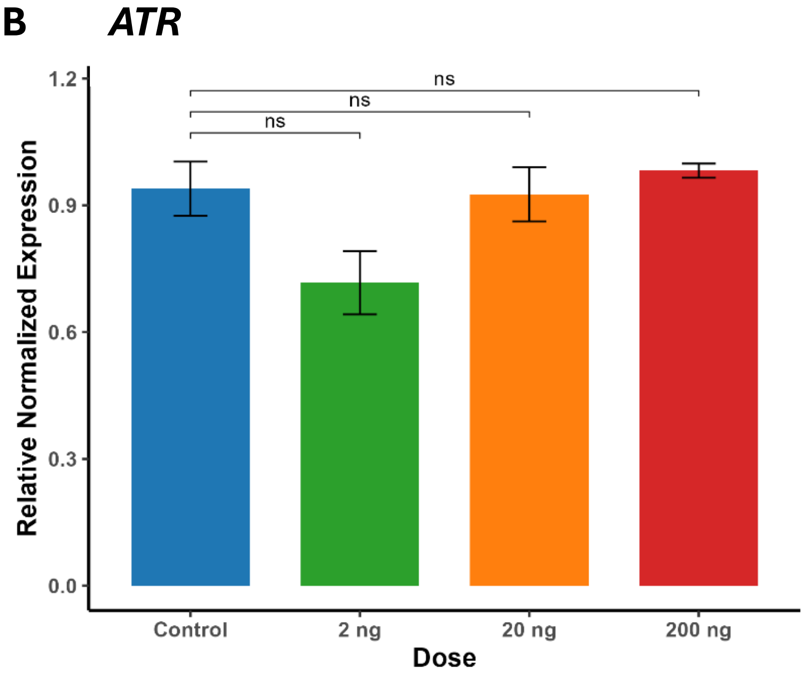
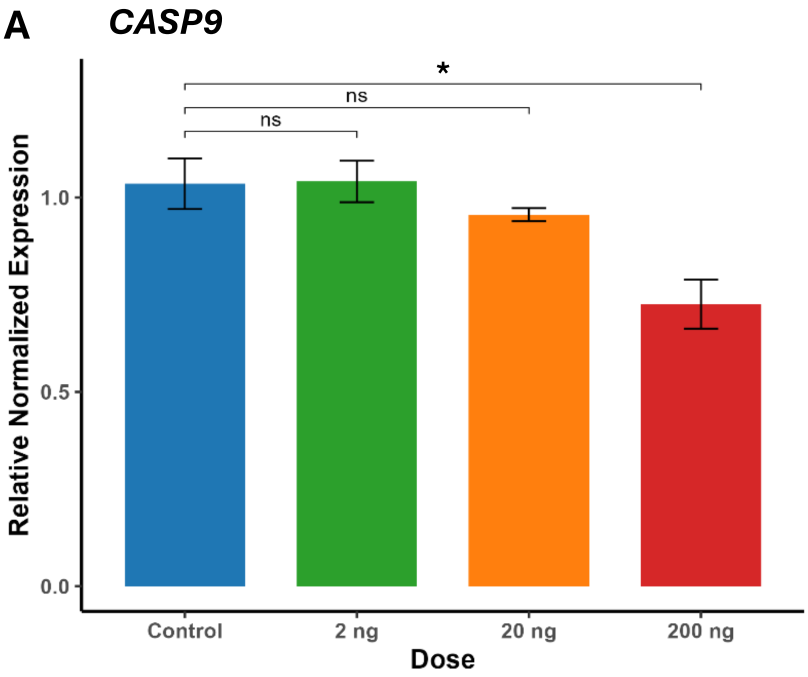

B
ATR
ns
ns
ns
Relative Normalized Expression
1.2
0.9
0.6
0.3
0.0
Control
2 ng
20 ng
200 ng
Dose

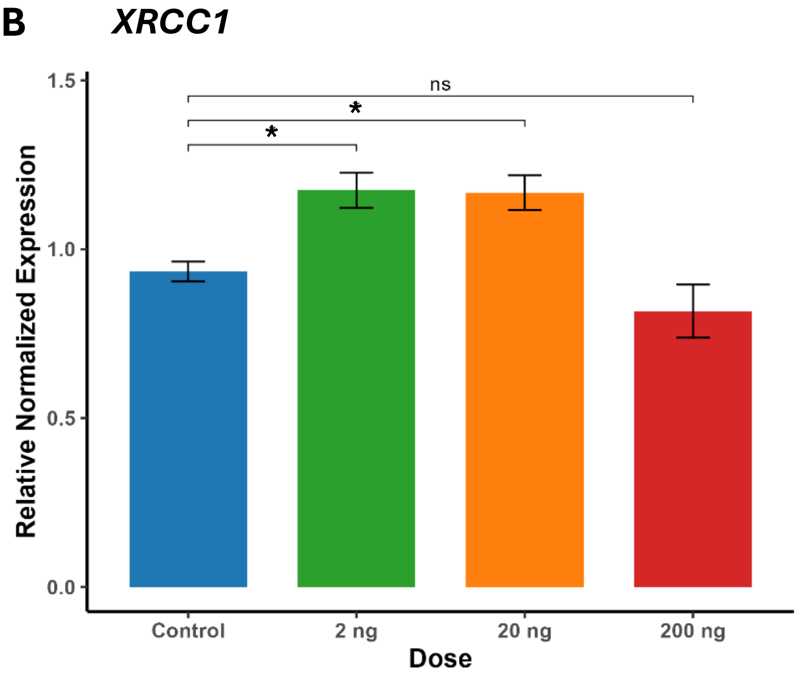
Figure 3.12. Gene Expression changes post fentanyl exposure in *ATM* and *ATR* in human Sertoli cells. To assess transcriptional responses of human Sertoli cells to escalating doses of fentanyl (0 ng/mL, 2 ng/mL, 20 ng/mL, 200 ng/mL), relative normalized gene expression was measured for: (A) *ATM* and (B) *ATR*. Each dose group consisted of 6 biological replicates (n = 6), and expression values were normalized to control. A Kruskal-Wallis test followed by Dunn's multiple comparisons test with Bonferroni correction was used to determine statistical significance between each fentanyl dose and control. All data are represented as mean ± standard error. ns indicates not significant, * is $p < 0.05$, ** is $p < 0.01$, and *** is $p < 0.001$.

A



CASP9
*
ns
ns
Relative Normalized Expression
1.0
0.5
0.0
Control
2 ng
20 ng
200 ng
Dose

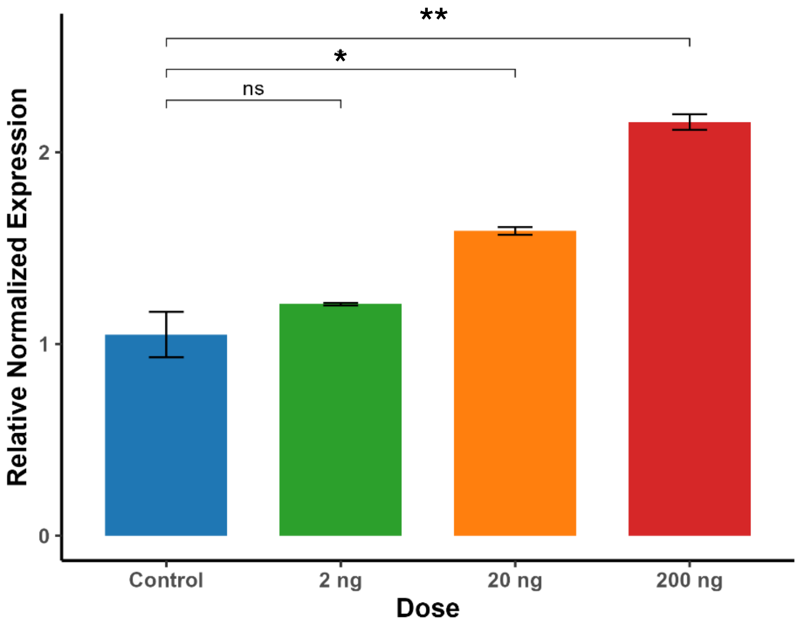
B



XRCC1
ns
*
*
Relative Normalized Expression
1.5
1.0
0.5
0.0
Control
2 ng
20 ng
200 ng
Dose

Figure 3.13. Gene Expression changes post fentanyl exposure in *CASP9* and *XRCC1* in human Sertoli cells. To assess transcriptional responses of human Sertoli cells to escalating doses of fentanyl (0 ng/mL, 2 ng/mL, 20 ng/mL, 200 ng/mL), relative normalized gene expression was measured for: (A) *CASP9* and (B) *XRCC1*. Each dose group consisted of 6 biological replicates ($n = 6$), and expression values were normalized to control. A Kruskal-Wallis test followed by Dunn's multiple comparisons test with Bonferroni correction was used to determine statistical significance between each fentanyl dose and control. All data are represented as mean ± standard error. ns indicates not significant, * is $p < 0.05$, ** is $p < 0.01$, and *** is $p < 0.001$.

OPRM1



**
*
ns
Relative Normalized Expression
0
1
2
Control
2 ng
20 ng
200 ng
Dose

Figure 3.14. Gene Expression changes post fentanyl exposure of *OPRM1* in human Sertoli cells. To assess transcriptional responses of human Sertoli cells to escalating doses of fentanyl (0 ng/mL, 2 ng/mL, 20 ng/mL, 200 ng/mL), relative normalized gene expression was measured for *OPRM1*. Each dose group consisted of 6 biological replicates ($n = 6$), and expression values were normalized to control. A Kruskal-Wallis test followed by Dunn's multiple comparisons test with Bonferroni correction was used to determine statistical significance between each fentanyl dose and control. All data are represented as mean ± standard error. ns indicates not significant, * is $p < 0.05$, ** is $p < 0.01$, and *** is $p < 0.001$.

3.5 DISCUSSION

Sertoli cells create a unique environment within the seminiferous tubules of the testis, supporting the development of male germ cells and in turn a main component of the blood-testis barrier (BTB). The BTB is constituted by different types of cellular junctions, such as tight junctions, gap junctions, desmosomes, and ectoplasmic specializations, all of which co-exist to form a physical barrier that separates the processes of spermatogenesis from the rest of the body and allows germ cell development to proceed in an immune-privileged state. Because of the intricate network of junctions that maintain this barrier's structure and function, changes in gene expression can alter how these proteins interact, ultimately disrupting barrier homeostasis. Considering the widespread and increasing use of synthetic opioids, namely fentanyl, the goal of this study was to determine whether exposure to physiologically relevant concentrations of fentanyl could alter the integrity of Sertoli cell tight junctions and compromise BTB function in our *in vitro* human model. Because concentrations of exogenous opioids remain poorly characterized in adult men involved in substance abuse, and exposure varies widely, we selected fentanyl concentrations according to serum-levels from clinical use. The results from these studies give plausibility that fentanyl may impair male reproductive health, namely by disrupting critical gene expression in Sertoli cells, inducing oxidative stress, and thus disrupting BTB permeability and resistance.

Fentanyl exposure elicited a paradoxical tightening of the BTB, evidenced by significantly increased transepithelial electrical resistance (TEER) and reduced permeability to small molecules. TEER is widely accepted as a quantifiable measure of tight junction integrity, with increased resistance indicating enhanced barrier function. The selective exclusion of small molecules suggests a tightening of the paracellular pathway, which may restrict essential solute exchange within the seminiferous epithelium. This hyper-tightening can be viewed as a compensatory response aimed at preserving barrier selectivity in response to the stress of opioid exposure. Similar compensatory remodeling responses have been reported following exposure to other toxicants in the blood-brain barrier (BBB), such as methamphetamine[226], and moreover a handful of toxicant substances have been found to alter BTB structure via different mechanisms[227]. Our results indicate that certain environmental challenges evoke a strengthening, rather than weakening, of the BTB. TEER was markedly elevated following each dose of fentanyl exposure, indicating an abnormal tightening of the Sertoli cell barrier rather than the expected decrease as seen in other barriers such as the BBB[228]. In endothelial models, the inhibition of glycogen synthase kinase-3β (GSK-3β), an anti-inflammatory promoter and modulator, has been shown to significantly increase TEER by stabilizing tight junction proteins like occludin and the claudin family, effectively prolonging their half-life and locking the barrier in a tightly sealed state[229]. By analogy, the elevated TEER in our human Sertoli cell model is consistent with a hyper stabilization of junctional complexes, suggesting an atypical strengthening of the BTB via anti-inflammatory pathways.

While typically a strong barrier prevents toxicant penetration, it is important to note that excessive tightening has the potential to disrupt physiological germ cell trafficking. Spermatogonial stem cell migration depends on finely tuned junctional remodeling[169,171,176,178]. Therefore, a non-dynamic, rigid barrier could impair critical spermatogenic processes. This concept is supported by the transcriptional upregulation we observed in tight junction genes such as *CLDN1*, *CLDN3*, and *CLDN7*, alongside stress-response regulators such as *OPRM1*, implying a cellular response aimed at barrier reinforcement under duress.

Interestingly, while TEER increased and small molecule permeability decreased, our assays showed no significant change in the permeability to larger molecules. This dissociation reflects the dual nature of tight junction regulation, which can be divided into the “pore pathway” (permits small ions/solutes) and the “leak pathpay” (permits larger molecules). Claudins primarily regulate the pore pathway, whereas leak-path permeability is influenced by dynamic remodeling of junction complexes and scaffolding proteins, and are also engaged with the actin cytoskeleton. Our data suggest that fentanyl exposure selectively modulates the pore pathway via claudins, while not significantly altering the integrity of the leak pathway. This nuanced response may reflect targeted cellular regulation aimed at preserving essential barrier selectivity while retaining capacity for controlled permeability to larger components like immunoglobulins or signaling molecules, implying that chronic opioid exposure may compromise tight junction selectivity without fully dismantling the BTB architecture. These selective permeability shifts could reflect altered regulation or disassembly of specific tight junction proteins, including claudins and occludins, which govern the passage of small molecules while excluding larger ones. These minor perturbations may stem from induced oxidative stress, cytoskeletal remodeling, or signaling changes in Sertoli cells[169,203,230]. While insufficient to allow large-molecule infiltration, this leakiness to small solutes could expose developing germ cells to toxicants or hormonal imbalances that are normally excluded by the BTB, potentially impairing spermatogenesis over time. Moreover, the lack of apparent large molecule permeability changes suggests that barrier compensation or partial recovery mechanisms may be active, preserving the overall scaffold of the BTB as a stress response. This disruption highlights the potential for early-stage BTB dysfunction that may precede more severe reproductive toxicity with prolonged or escalated opioid use. Notably, opioids have been shown to be transported across the blood-testis barrier (BTB) via various drug transporters, including efflux pumps like P-glycoprotein (P-gp), which can limit the entry of opioids into the testes[231]. Conversely, some studies suggest that certain drug transporters may facilitate the movement of opioids across the BTB, potentially impacting spermatogenesis and male fertility, as endogenous opioid peptides have been suggested to inhibit Sertoli cell function in an autocrine and paracrine manner[123].

Despite dose-dependent changes in oxidative stress and gene expression, fentanyl exposure at 2 ng/mL, 20 ng/mL, and 200 ng/mL over our chronic dosing period did not significantly alter apoptotic populations in our human Sertoli cell model, as measured by Annexin V staining. These findings suggest that fentanyl, under the dosing conditions used in this study, does not directly trigger apoptotic cell death in this somatic cell population, at least initially. Interestingly, oxidative stress assays demonstrated significantly elevated reactive oxygen species (ROS) production at moderate and high doses. Oxidative stress is a known indicator of many negative outcomes in cellular health. Notably, it has also been implicated as a disruptor of tight junction stability and is closely linked to the disruption of barriers in other models. Fentanyl exposure led to a significant, dose-dependent increase in ROS^+ Sertoli cells without a corresponding rise in apoptotic populations, indicative of sub-lethal oxidative stress. This is noteworthy considering that ROS is commonly implicated in the initiation of apoptotic signaling cascades. Our results indicate that oxidative stress can activate other cellular signaling pathways without triggering cell death across each dose group. Our dosing range was chosen to be comparable to clinically relevant doses of fentanyl used in medical procedures and post medical treatment and thus were predicted be sublethal even at the highest dose. However, we expected to potentially see an increase in apoptotic populations based on the current findings within the literature. Additionally, the 10-day exposure period may have allowed time for adaptive responses to develop. Chronic but non-acute oxidative stress can lead to cytoprotective gene expression, cytoskeletal remodeling, and junctional reorganization, without necessarily activating intrinsic apoptosis pathways.

Our results align with broader literature showing that opioids generate ROS across a wide array of cell types, however, downstream effects seemingly vary based on the context. For example, chronic administration of tramadol and morphine elevates oxidative stress and can lead to apoptosis in neural tissues[217,232], however, opioid receptor engagement has also been shown to suppress LPS-induced ROS production via Nrf2/HO-1 activation in microglia[233] highlighting a potential dual role depending on specific cellular context. Notably, in testicular models, morphine disrupts Sertoli cell function via oxidative and apoptotic mechanisms, which can be mitigated by zinc through p53/Akt pathway modulation[234]. In contrast, our findings report no apoptosis despite heightened ROS in our human Sertoli cells, suggesting a fluctuating, adaptive barrier response to fentanyl not characterized by cytotoxicity. This selective ROS-driven enhancement of tight junctions, without apoptosis, suggests that Sertoli cells engage oxidative signaling for homeostatic reinforcement of the BTB under opioid stress—a mechanism distinct from the cytotoxic outcomes observed in other opioid-exposed tissues. In our BTB model, this oxidative stress correlated with increased TEER and reduced paracellular flux of small molecules, suggesting ROS-promoted tight junction reinforcement. Indeed, ROS-mediated activation of MAPK pathways (p38 MAPK) has been shown to enhance claudin expression and tighten junctional complexes in endothelial and epithelial models[235,236]. Importantly, the absence of increased cell death in our model confirms that fentanyl-induced ROS remained below apoptotic-triggering thresholds, while still leading to alterations in barrier permeability and resistance. Comparable phenomena have been observed in endothelial models, where moderate ROS elevation disrupts tight junction protein localization via p38 MAPK activation without inducing apoptosis as previously described. In our model, the increased oxidative stress correlates with increased TEER and reduced small-molecule flux, indicating a selective tightening of the pore pathway. This is consistent with the concept that ROS can modulate tight junction architecture, particularly claudin-mediated paracellular routes, via post-translational modification or transcriptional regulation of junctional proteins. Importantly, we observed a sustained increase in TEER five days after cessation from fentanyl across each dosing group, indicating that these alterations to the BTB may have lasting effects outside of the noted apoptosis after opioid exposure.

Collectively, these results suggest a nuanced model in which opioid exposure in Sertoli cells modulate signaling that strengthens the barrier by upregulating tight junction components (e.g. claudin-1, claudin-3, claudin-7), rather than causing dysfunction or cell death initially. While this unique, adaptive, sub-apoptotic oxidative stress response indicate a protective mechanism, it is important to note that this tightening of the BTB due to ROS may also be an issue for male reproductive health and spermatogenesis. As mentioned, this "locking" of the BTB may play a role in the consequences observed in men who use opioids via non-apoptotic means. Spermatogonia and preleptotene spermatocytes must cross the

BTB to continue development, and excessive tightening may trap them inappropriately, impairing spermatogenesis[172]. Furthermore, maturing sperm may be unable to exit into the lumen, thus leading to decreased sperm counts and reduced fertility. Additionally, an increase in barrier rigidity may affect nutrient and waste exchange, along with paracrine signaling between Sertoli and germ cells, and thus isolated from hormonal, metabolic, or immune signals necessary for function or repair, potentially disrupting spermatogenesis.

To further investigate this phenomenon, we conducted RT-qPCR gene expression analysis, identifying several genes whose expression was significantly altered via opioid exposure. Overall, these findings suggest that fentanyl interferes with key cellular pathways involved in BTB integrity, stress response, and junctional signaling, providing mechanistic insight into opioid-induced testicular dysfunction. Notably was the observation of significant upregulation of *CLDN1*, *CLDN3*, and *CLDN7* at higher fentanyl doses. These genes encode claudins, integral membrane proteins that form tight junctions critical to the BTB[165,169,237]. The BTB compartmentalizes the seminiferous epithelium, protecting developing germ cells from autoimmune attack and toxic insults. Increased expression of these claudins may represent a compensatory response to BTB disruption or a pathological tightening of the barrier that impairs necessary paracellular trafficking. Interestingly, *CLDN1* and *CLDN3* are also involved in the regulation of epithelial permeability and cell polarity. In non-testicular systems, opioid exposure has been shown to disrupt tight junction architecture, including *ZO-1* and occludin, through *MAPK* and oxidative signaling pathways[57,235]. The observed upregulation may therefore reflect opioid-mediated activation of stress or inflammatory signaling, leading to transcriptional upregulation of junctional genes to preserve barrier function, but as mentioned may hinder spermatogenesis.

It is well understood that tight regulation of redox balance is critical for cell health and function. We observed significant changes in the expression of *CAT* (catalase) and *SOD1* (superoxide dismutase 1)[238], both of which play central roles in detoxifying reactive oxygen species (ROS). Catalase decomposes hydrogen peroxide into water and oxygen, while *SOD1* converts superoxide radicals into less reactive intermediates. The downregulation of *CAT* at low fentanyl doses and compensatory upregulation at higher doses suggest a biphasic oxidative stress response, characterized by an initial protective or adaptive phase followed by a detrimental phase when oxidative stress becomes excessive. This means that low levels of oxidative stress may potentially trigger beneficial cellular responses, like activating antioxidant defenses or promoting cell survival. However, high levels of oxidative stress imply an overwhelming response, leading to cellular damage and potentially disease. This may reflect increasing ROS generation via opioid interference with mitochondrial metabolism and cellular respiration in Sertoli cells.

Low *SOD1* expression and thus activity can leave cells vulnerable to oxidative stress due to inadequate metabolism of O_2. Increasing *SOD1* activity, however, is expected to be protective, and if increased beyond a certain threshold, its protective effect is lost and can even lead to cellular damage by producing excessive H_2O_2[239]. Therefore, *SOD1* function requires the correct threshold to provide optimal protection against oxidative stress. Both insufficient and excessive *SOD1* activity can have detrimental consequences. In our study, we observed an increased *SOD1* expression in our low (2 ng/mL) and medium

(20n ng/mL) dosing groups, however interestingly enough, our highest dose (200ng/mL) featured a significant increase in expression comparable to our low dose. While a dose-dependent response could be expected, this could be due to *SOD1* activity being regulated in response to opioid-induced oxidative stress, or alternatively, high doses of fentanyl contributing to oxidative stress and subsequently disrupting *SOD1* function via overwhelming ROS activation.

Nonetheless, chronic oxidative stress is known to impair spermatogenesis, damage DNA, and disrupt BTB function[180,240,241]. These results align with previous studies reporting fentanyl-induced oxidative stress-related damage in other tissues. Here, we provide insight into opioid-induced oxidative stress at sub-apoptotic levels in human Sertoli cells *in vitro*, leading to changes in gene expression and BTB alterations that may be implicit in opioid-induced infertility or reproductive disruption.

SOX9, GATA4, and *AR* (androgen receptor) are essential regulators of Sertoli cell differentiation, development, and hormonal responsiveness. *SOX9* plays a key role in the differentiation of Sertoli cells, which are essential for testis development in males. *GATA4* is a transcription factor that modulates genes involved in testicular development and steroidogenic support, while *AR* mediates the actions of testosterone, a key driver of Sertoli cell function, fertility, and spermatogenesis[242,243]. Although changes in gene expression in both *SOX9* and *AR* were not statistically significant at medium and high doses of fentanyl in our study, both showed significant decreases at our lowest dose (2 ng/mL). Decreased *SOX9* expression can have a variety of effects, including impacts on cell proliferation, differentiation, and survival, depending on the specific context and cell type[244,245]. In some cancers, reduced *SOX9* expression is associated with a better prognosis, while in others, it can indicate a poorer outcome. Whether or not this significant decrease at low doses of opioid exposure is relevant warrants further investigation. Given its role in the development and maturation of Sertoli cells early in sexual maturation, it may be assumed that *SOX9* expression, and thus testicular development, could be significantly impacted via early life exposure to exogenous opioids. *AR* plays a crucial role in male reproductive function and is essential for sperm production among other aspects of male fertility, and it is widely accepted that the requirement of testosterone for spermatogenesis is mediated by somatic cells in the testis, including Sertoli cells.[246]. If *AR* expression is reduced in the testes, it can lead to lower sperm production and potentially decreased fertility[247].

While a decrease was only observed at the lower dose of fentanyl, *AR*, like *SOX9*, could indicate vulnerability in earlier developmental stages, although more research is required to fully understand this notable decrease. *GATA4* was significantly altered, noting a dose-dependent response to opioids across each group. Researchers have determined that *GATA4* plays a pivotal role in the regulation of BTB function[242]. Previous research had observed a reduction in GATA4 in Sertoli cells was linked with reduced levels of tight junction proteins and accompanied by a loss of morphologically recognizable junctional complexes and a decline in epithelial membrane resistance[248]. Here, we observe an increase in GATA4 expression, and an increase in epithelial barrier resistance, which aligns with these prior findings. Collectively, our results may suggest potential disruption in the androgenic signaling axis, which may further compromise BTB function and germ cell support. As mentioned, scientists have linked opioid exposure to hypogonadism and disrupted hypothalamic-pituitary-gonadal (HPG) axis function, and this data supports these effects outside of crosstalk with the endocrine system, and instead directly at the testicular level in our human *in vitro* model. Further research is required to determine if these observed gene expression changes occur comparably *in vivo*, and if these changes have greater impacts on Sertoli cell and male reproductive development in early life.

ATM, *ATR*, *CASP9*, and *XRCC1* are DNA repair genes critical for sensing and responding to genotoxic stress[249-251]. *ATM* and *ATR* are serine/threonine kinases that initiate cell cycle checkpoints and DNA repair following double strand breaks or replication stress, respectively. Opioids have been shown to induce DNA damage in regions of the brain, including double strand breaks[252]. While we observed nonsignificant differences in *ATR* gene expression from fentanyl exposure in our study, there did appear to be a notable trend in dose and expression. Interestingly, *ATM* expression was significant at our medium dose of fentanyl (20 ng/mL), indicating a possible molecular signaling response to DNA damage arising from opioid exposure. *CASP9* plays a significant role in the initiation of apoptotic pathways within the testis, particularly in the context of male germ cell apoptosis. Our results indicated a nonsignificant trend in declining gene expression alongside increasing doses of fentanyl, until reaching significance at the highest dose (200 ng/mL). Interestingly, one study had shown that the failure of activating the caspase-9 pathway induces a higher cellular threshold for cisplatin-mediated induction of apoptosis in testicular cancer cells[253]. Perhaps chronic opioid use at high concentrations has the potential to regulate apoptotic signaling pathways such as *CASP9*, and given our Annexin V assay results, warrants further investigation. *XRCC1* plays an important role in single-strand and double-strand break repair as well as base excision repair and is highly expressed in germ cells due to their vulnerability during development. In human Sertoli cells, *XRCC1* was also found to be expressed, unlike in other quiescent somatic testicular cells [254]. This may be of relevance to the BTB's dynamic regulatory measures in protecting the sensitive development of germ cells, and a necessary function to endure potential genotoxic damage. We observed significantly increased *XRCC1* expression at our low and medium doses, however not at our highest dose. The altered expression of these genes may indicate fentanyl-induced genotoxicity in Sertoli cells. Perhaps at higher doses, a different repair mechanism mediates DNA damage, although further studies are required to determine this observation. Given that opioids can increase ROS and have been implied to impair mitochondrial respiration, the accumulation of oxidative damage could contribute to DNA damage, and in turn activate these pathways.

Interestingly*,* *OPRM1*, the gene encoding the mu-opioid receptor (MOR), was significantly altered in response to medium and high doses of fentanyl. MORs are G-protein coupled receptors that mediate the cellular effects of endogenous and exogenous opioids, including analgesia, sedation, and hormonal modulation[74], and traditionally, chronic opioid use is generally associated with downregulation of *OPRM1* and speculated to be due to epigenetic modifications like hypermethylation. However, the response can be complex and influenced by the duration and dose of exposure, as well as the individual's genetic background[255]. In testicular tissue, MOR expression has been observed in both germ and Sertoli cells, and its activation is known to impair testosterone synthesis and induce apoptosis[180,256]. The upregulation of *OPRM1* in Sertoli cells may reflect a feed-forward mechanism where opioids either sensitize or desensitize receptor signaling and thus mitigate modulation of the receptor. It has been suggested that there are multiple mechanisms that contribute to opiate-induced spermatogenic disruption and testicular disruption. However, these results indicate that *OPRM1* is upregulated, at least within our dosing timeframe of 10 days, and therefore hypermethylation and the subsequent reduced gene expression was not observed in our model. Together with the findings in this study, these results suggest a potential autocrine or paracrine feedback loop in which exogenous opioid exposure alters the expression of its own receptor within the human Sertoli cells of our human *in vitro* BTB model. Whether or not this is indicative of an increase due to initial exposure and subsequently followed by hypermethylation, and thus declined expression, or a Sertoli-specific regulatory mechanism to prevent hypermethylation, remains to be determined and requires more investigation.

Overall, the blood–testis barrier (BTB) is one of the most structurally and functionally unique barriers in the body. Unlike the blood–brain barrier (BBB), which is formed by endothelial cells supported by astrocytes and pericytes, the BTB is formed exclusively between adjacent Sertoli cells and consists of a complex array of junctions and features that create an immunoprotected microenvironment essential for germ cell development. The BTB is also exceptionally dynamic, undergoing cyclical restructuring to allow germ cell movement without compromising barrier integrity; a property not observed frequently in other barriers. Our findings that fentanyl exposure significantly increased transepithelial electrical resistance (TEER) and reduced paracellular permeability to small molecules align with the BTB's physiological dynamic and selective nature. Elevated expression of *CLDN1*, *CLDN3*, and *CLDN7* further supports these findings, as these transmembrane proteins are central to Sertoli cell tight junction function and are known to be regulated by hormonal and environmental stimuli[169,181,237,257,258]. Importantly, this selective enhancement of the barrier resembles the behavior of other tissue barriers under stress, and although barrier disruption is typically described in a decreased barrier resistance and increased permeability, we observe the opposite, indicating potentially a regulatory response due to stress induced by endogenous opioid exposure. Importantly, this tightening of the barrier may also have negative impacts on the BTB, and thus spermatogenesis and male reproductive health via disrupting the movement of important nutrients and molecules for germ cell development, as well as restricting germ cell movement into and through the lumen of the seminiferous tubules.

Changes in gene expression levels were noted for oxidative stress as well as DNA repair mechanisms, indicating potential repair mechanisms activating in response to chronic opioid use at doses that did not exhibit apoptotic pathway activation. Together, these results highlight the BTB's unique ability to mount a compensatory, selective response to opioid-induced stress, mirroring aspects of other barrier systems while retaining its specialized regulatory functions in the testis.

The observed alterations are plausible mechanisms for the adverse effects of opioid exposure on male testicular function and reproductive health. Consequently, opioid-induced hypogonadism may not only arise from disruptions to the endocrine system, but also from a direct impact Sertoli cells and the BTB. These findings give evidence to this direct effect of opioid use on the male reproductive tract and indicate that opioid-induced alterations in fertility or gamete health may be in part due to disruptions to this highly specialized somatic niche and the cells within. This study also provides novel, mechanistic insight into how exogenous opioid use may disrupt key cellular systems that maintain human spermatogenesis and protect the testicular microenvironment.

3.6 METHODS & MATERIALS

Human Sertoli Cell Isolation

Human Sertoli cells were originally isolated from de-identified human testicular cell suspensions by Dr. R. Clayton Edenfield and provided by Dr. Kyle Orwig at the University of Pittsburgh (Sample ID: Human Core – 18092304). Suspensions were cryopreserved in liquid nitrogen at a concentration of ~20 × 10^6 cells/mL until use. Upon thawing, germ cells were separated by gravity sedimentation in 10 mL of HBSS containing penicillin/streptomycin (P/S). The resulting cell pellet underwent enzymatic digestion in a shaker incubator at 300 rpm and 37°C for 5 minutes using a digestion solution consisting of 80 mL HBSS + P/S, 10 mL trypsin (2.5%), 10 mL collagenase type IV (1%), and 2 mL DNase (1 mg/mL). Digestion was halted with 0.1% soybean trypsin inhibitor. The suspension was centrifuged at 200 × g for 5 minutes, the supernatant was discarded, and the pellet resuspended in DMEM/F12 with P/S at a 10:1 ratio. This mixture was incubated at room temperature for 30 minutes to separate tubular fragments (pellet) from interstitial cells (supernatant). Interstitial cells were further purified using a discontinuous Percoll gradient. To remove peritubular cells, the tubular cell pellet was treated with PBS containing 1M glycine and 2 mM EDTA and pipetted for 10 minutes. Sertoli cells were then enriched via sedimentation and plated alongside tubular fragments in DMEM/F12 supplemented with 15% FBS and P/S. Cultures were maintained with media changes every other day. Sertoli cells were further purified through visual inspection and differential plating. At the third passage, cells were cultured at 35°C in DMEM/F12 with 10% FBS, 5 mL P/S, 5 mL GlutaMAX, and supplemented with follicle-stimulating hormone (FSH, 1:1000) and epidermal growth factor (EGF, 1:2000). Medium was refreshed every other day throughout the culture period.

Human Sertoli Cell and TRT Human Sertoli Cell Culture

Both TRT and non-TRT human Sertoli cells were cultured at 35°C in DMEM/F12 with 10% FBS, 5 mL P/S, 5 mL GlutaMAX, and supplemented with follicle-stimulating hormone (FSH, 1:1000) and epidermal growth factor (EGF, 1:2000). Medium was refreshed every other day throughout the culture period and passaged after reaching 90% confluence.

Opioid Treatment

The Sertoli cells were treated Fentanyl Citrate (MilliporeSigma) generously provided by Dr. Franklin West, at concentrations reflecting plasma levels in healthy adult men ranging from 0 – 200 ng/mL. Confluent cultures were treated with fentanyl at concentrations of 2 ng/mL, 20 ng/mL, or 200 ng/mL. The cells were maintained in 10% FBS DMEM media with fentanyl or water-only vehicle controls, with media changes occurring every two days. Both the isolated and TRT human Sertoli cells were grown to 95% confluency, then trypsinized and seeded in 12 mm Transwells (Costar, Corning® Life Sciences) coated with Matrigel (Corning® Life Sciences) at 1×10^6 cells/cm^2 per well, for continued growth for 10 days in fentanyl-containing media or control media before Annexin V, Oxidative Stress, Transepithelial electrical resistance (TEER), barrier permeability assays, and RT-qPCR gene expression studies were conducted.

Cell Viability & Oxidative Stress Assays

By utilizing the Muse® Annexin V and Dead Cell Assay Kit (Luminex), cell viability was assessed by measuring the percentage of apoptotic cells in the cultures by staining unfixed cells with Annexin V and 7-AAD as per manufacturer’s instructions in preparation for flow cytometry. Each sample was analyzed on the Muse® benchtop flow cytometer (MilliporeSigma) and analyzed at 1,000 events for three replications (n = 3) per opioid concentration and medium-only control. The Muse® ROS Oxidative Stress Assay Kit, Oxidative Stress was determined by measuring the percentage of ROS+ cells in the cultures per manufacturer's instructions in preparation for flow cytometry. Each sample was analyzed on the Muse® benchtop flow cytometer (MilliporeSigma) and analyzed at 5,000 events for four replicates (n = 4).

Transepithelial Electrical Resistance Measurements

The functionality of the BTB was assessed by performing transepithelial electrical resistance (TEER) and barrier permeability assays. Confluent cultures were trypsinized and seeded at 1×10^6 cells/cm^2 in the apical (upper) chamber of the 12 mm diameter Transwell (Costar) coated with Matrigel® (Corning®), as described above. The next day (Day 0), the TEER of the cells in was measured using an Epithelial Voltohmmeter (EVOM, World Precision Instruments, Inc.) in ohms (Ω) × cm^2, as previously described to determine healthy cell-barrier formation. After the measurement, the cells were treated with clinically relevant doses of either fentanyl or water (control), 10 µM testosterone (Sigma), supplemented with follicle-stimulating hormone (FSH, 1:1000) and epidermal growth factor (EGF, 1:2000). The TEER measurements were recorded, and media changes occurred every other day for 10 days.

Paracellular Flux Permeability Assay

After the TEER was measured on the 10th day, the fentanyl-treated human Sertoli cells were equilibrated with Ringer's solution for 15 minutes at 35°C, and then the apical chamber was replaced with Ringers and 50 µg/mL Texas Red-labeled dextran (10 kDa;

ThermoFisher) and 2 µg/mL calcein (0.63 kDa; ThermoFisher) as described. Over the course of two hours, 100 µL of media was collected from the basal side of the chamber every 30 minutes and used for measuring the intensity of fluorescence at wavelengths Ex485/Em525 nanometers for calcein and Ex585/Em625 nanometers for dextran using a SpectraMax iD5 microplate reader (Molecular Devices). The amount of flux was determined by a standard curve and was graphed as the amount of calcein or dextran appearing in the lower chamber of the Transwell plotted versus time. The rate of diffusion of calcein and dextran was calculated using the slope of the flux curve as previously described.

RNA Extraction & Quantification

RNA was extracted following the manufacturer's instructions from the fentanyl-treated or control TRT human Sertoli cells using the RNeasy Plus Mini Kit (Qiagen) after the TER was measured on the 10th day to yield three biological replicates (n = 3). Using the NanoDrop® 2000c spectrophotometer (Thermo Scientific), RNA integrity was measured, and only 260/280 ratios >2.0 were accepted for further processing. The samples were stored at -80°C until real-time quantitative polymerase chain reaction analysis and stored for later use.

Real-Time Quantitative Polymerase Chain Reaction RT-qPCR Analysis

Each RNA sample (500 ng) was reverse transcribed to cDNA for RT-qPCR using the iScript™ cDNA Synthesis Kit (Bio-Rad) as per the manufacturer's instructions. Quantitative PCR was performed with 500 µg cDNA and the iQ™ SYBR Green Supermix (Bio-Rad) as per manufacturer's instructions using the CFX Connect Real-Time PCR Detection System (Bio-Rad). The amplification parameters were as follows: the initial denaturation of 3 min at 95°C, 40 cycles of 15-second denaturation at 95°C and 1 minute at 57°C for annealing and extension. After the final PCR cycle, a melt curve analysis was performed at the following parameters: 5 seconds per step, 65°C to 95°C at 0.5°C increments.

All PCR primers (Integrated DNA Technologies) were designed and validated for specificity and amplification efficiency to human genes *GAPDH*, *OPRM1*, *CLDN1*, *CLDN3*, *CLDN7*, *SOX9*, *GATA4*, *AR*, *ATM*, *ATR*, *CASP9*, *CAT*, *SOD1*, and *XRCC1*. *GAPDH* was used as an internal control for normalization. For each experiment, there were three replicates for each gene, and two plates were run per vehicle control, and analgesic concentration representing 3 separate and distinct biological replicates (n = 6).

Statistical Analysis

R-Studio with R (version 4+) was used for data analyses and modeling. Microsoft Excel was used for data organization and graphical representations. Both were used to analyze cellular apoptosis, oxidative stress, TEER, paracellular dye flux, and RT-qPCR data. TEER, Annexin V/Dead Cell Assay, and Oxidative Stress (ROS) Assay were analyzed with one-way analysis of variance (ANOVA) and Tukey's post hoc test, where * is $p < 0.05$, ** is $p < 0.01$, and *** is $p < 0.001$. Significant differences for the paracellular flux assays were determined by a two-way analysis of variance (ANOVA) with Dunnett's correction for multiple comparisons, where * is $p < 0.05$, ** is $p < 0.01$, and *** is $p < 0.001$. RT-qPCR gene expression analyses were calculated by the ΔΔCT method as normalized fold differences in each target gene using BIORAD CFX Maestro Software and PCR instruments.

CHAPTER 4

INVESTIGATING THE IMPACTS OF EXOGENOUS OPIOIDS ON SPERMATOGENESIS AND THE SPERM EPIGENOME

4.1 INTRODUCTION

The opioid epidemic constitutes one of the most pressing public health crises of the 21st century, characterized by a dramatic increase in opioid consumption, dependency, and overdose-related mortality, particularly in North America. Initially driven by the overprescription of opioid analgesics during the late 1990s and early 2000s, the crisis has since escalated with the proliferation of illicit synthetic opioids such as fentanyl, which exhibits potency up to 100 times greater than morphine. This shift has led to a profound rise in mortality, with opioids now accounting for the majority of drug overdose deaths in the United States. While the immediate consequences of opioid use are widely acknowledged—namely, addiction and fatal overdose—the broader implications for public health extend far beyond acute outcomes. Notably, the epidemic disproportionately affects individuals of reproductive age, particularly men, raising urgent questions about its impact on fertility, prenatal development, and transgenerational health. These concerns are compounded by global trends indicating declining sperm counts and fertility rates, prompting critical examination of environmental and pharmaceutical contributors to male reproductive dysfunction.

Historically, many chronic diseases have been attributed to genetic predisposition or otherwise classified as idiopathic when the etiology was otherwise unclear. However, modern research has increasingly recognized the role of environmental exposures, including pharmaceuticals, in shaping long-term health. This paradigm shift is underpinned by the emergence of epigenetics and the interaction of environmental and genetic factors contributing to health and disease, which together offer a powerful model for understanding how exposures during sensitive developmental windows can influence disease risk across the lifespan.

Epigenetics refers to heritable modifications in gene expression that occur independently of changes in the underlying DNA sequence. These modifications include DNA methylation, histone post-translational modifications, and regulatory non-coding RNAs[25,259,260]. The epigenome is particularly plastic during early development and germ cell maturation, rendering it highly responsive to external cues such as nutritional status, toxicants, stress, and pharmacological agents. Originally conceptualized by Conrad Waddington in 1942, the field of epigenetics has become central to the DOHaD hypothesis, which posits that environmental factors encountered during critical periods of development can permanently alter physiological trajectories, thereby modulating risk for a range of adult-onset diseases, including metabolic, cardiovascular, and neurodevelopmental disorders[25].

Importantly, emerging evidence suggests that epigenetic changes induced by environmental exposures are not confined to somatic outcomes but may also affect the germline, with the potential to transmit altered disease susceptibility across generations.

This recognition highlights the importance of paternal exposures, which have historically been underappreciated in assessments of reproductive and developmental risk[28], and require much needed investigation.

In the context of the ongoing opioid epidemic, recent studies have begun to elucidate the adverse effects of opioid compounds, both natural and synthetic, on male reproductive health. Although this area of research remains nascent, current findings implicate opioids in the disruption of key physiological processes within the male reproductive tract, including hormonal regulation, spermatogenesis, and the integrity of the blood-testis barrier[38,102,113,124,210]. Given the high prevalence of opioid use among men of reproductive age and the increasing potency of available substances, there is an urgent need to expand research efforts in this domain. A deeper mechanistic understanding of opioid-induced reproductive toxicity will be critical to informing public health interventions and regulatory strategies aimed at mitigating any long-term consequences of opioid exposure, although investigating reproductive health and fertility in humans, let alone men and substance abuse, pose uniquely distinct challenges.

Notable studies have demonstrated that chronic opioid use leads to decreased sperm counts and semen parameters, with particular focus on reduced motility, abnormal morphology, and increased DNA fragmentation[111]. Further studies have also found that opiate-dependent men had sperm concentrations ~22 million/mL versus 66 million/mL in controls, paired with significantly higher DNA fragmentation (36.4% vs. 27.1%)[212] and highlighted that these observations persisted even after accounting for opioid-induced hypogonadism, suggesting a potential direct effect on the testes that may have long-term effects on spermatogenesis.

The μ-opioid receptor gene *OPRM1* encodes the primary receptor responsible for both the rewarding and analgesic effects of endogenous opioids (e.g., β-endorphins) and exogenous opioids (e.g., morphine, fentanyl)[65,105]. Extensive research has been done regarding this receptor, particularly within the nervous system and its role in addiction. In *OPRM1*, a single nucleotide polymorphism (SNP), A118G, has been extensively studied and associated with an increased risk for opioid addiction across diverse populations[261]. In a multi-trait genome-wide association study, this SNP demonstrated the strongest genome-wide association signal for opioid addiction, supporting the notion of *OPRM1* as a key genetic risk locus[262]. Alongside opioids, both *OPRM1* and this polymorphism have been implicated in other substance abuse and addiction behaviors[263,264]. Beyond genetic variation, epigenetic modifications of *OPRM1*, particularly DNA methylation at its promoter region, are implied to be a key feature of modulating opioid receptor function and expression, thus playing a role in addiction and dependence[265]. Furthermore, short-term fentanyl exposure during surgery was shown to produce a 26% global DNA hypomethylation and suppressed expression of methyltransferase genes DNMT1a (−68%), DNMT3a (−65%), and DNMT3b (−71%)[266]. Alterations in DNA methylation have been detected notably long after the use of prescription opioids[110], leading researchers to believe it could have lasting effects that may contribute to negative health outcomes observed in those who abuse opioids, and potentially unknown outcomes later in life.

Interestingly, evidence suggests that the endogenous opioid system is involved in the regulation of male reproductive function. Studies have shown the presence of endogenous opioid peptides (EOPs) alongside their respective opioid receptors in different organs and tissues within the male reproductive system indicates that EOPs likely participate in some role of reproductive homeostasis[123]. Opioids are known to interact in various organs and tissues within the body, including the endocrine system. Regarding the HPG axis, opioids have been shown to decrease not only LH but also testosterone and estradiol, which have effects on testicular function[103,124-126]. This interaction is suspected to play a role in the regulation of both the HPG axis and the reproductive system.

Conversely, exogenous opioid use, like fentanyl and morphine, has been shown to induce hypogonadism paired with decreased libido and erectile dysfunction in men chronically abusing these substances, as well as inducing infertility[124]. These findings collectively indicate that EOPs can regulate reproductive function by inhibiting the secretion of GnRH at the level of the central nervous system[127,128]. Studies have demonstrated presence of EOPS in different testicular cell types alongside the presence of their respective opioid receptors in animal testes[129-132], and suggests both indirect and direct involvement in male reproductive function. Despite these negative impacts observed in men, little research has examined direct involvement from exogenous opioids on the somatic niche and spermatogenesis.

Studies have also discovered endogenous opioids and their receptors within male germ cells[135,136]. These findings suggest that opioid receptors may be expressed during spermatogenesis and potentially be important in either cell maintenance or function. With the male reproductive process being highly complex and sensitive, it can be said that any disruptions or exposures, at any critical point during this process, have the potential to induce negative consequences. Given that endogenous opioids and receptors are present within the male reproductive tract, including germ cells, exogenous opioid use has the potential to lead to severe disruptions including impaired fertility, hormone production, alterations in sperm production and development, and semen parameters.

Importantly, the development of germ cells also requires dynamic DNA methylation, and moreover, one study had found that men with opioid dependence exhibit significant *OPRM1* promoter hypermethylation in both blood and sperm, particularly at CpG site 2[115,198]. While the DNA methylation levels were overall higher in blood, expression levels of this receptor are comparatively lower elsewhere in the body, and this observation sparked interest in the possibility of germline epigenetic modulation induced by opioids.

While there is evidence of opioid involvement in the alteration of both histones and miRNAs, there are few but noteworthy studies investigating changes in DNA methylation in the context of opioid use[267]. One study examining the leukocytes from opioid users showed an increase in methylation at a CpG rich island in the *OPRM1* gene coding for the mu opioid receptor and at the long interspersed nuclear element (LINE-1) compared to controls[268]. LINEs are non-long terminal repeats and part of the retrotransposon family, which are mobile genetic elements that utilize germline copy mechanisms to spread throughout the genome[269]. Notably, global methylation sites are especially prevalent at these LINE-1 elements. Although the *OPRM1* methylation had no direct effect on MOR transcription levels, high levels of chronic pain were substantially correlated with the global DNA methylation at LINE-1 in another study [267]. Considering that male germ cells both express *OPRM1* and are speculated to be modulated by endogenous opioid signaling in the male reproductive process, the idea of more potent exogenous opioids inducing epigenetic alterations, specifically through DNA methylation, demands further investigation.

Mammalian germ cells undergo waves of methylation remodeling, necessary for imprinting and element silencing to establish the correct epigenetic state for reproductive success. There is evidence that disruptions in this establishment can lead to harmful consequences, as previously described[1,190,193,196,270]. Therefore, epigenetic interference from opioids during these critical windows may induce persistent germline errors, increasing the risk of subfertility and genetic inheritance of epimutations.

4.2 PURPOSE OF THE STUDY

A growing body of evidence suggests there may be negative consequences induced by exogenous opioids on male gametes and spermatogenesis. Concerns regarding opioid use among men of reproductive age has risen alongside the ongoing opioid crisis. Opioid-induced methylation changes could impair fertility and compromise offspring health, through epigenetic modulation and therefore disruption of germ cell function. While evidence exists for opioids to induce potential alterations in this manner, the purpose of this study aims to determine if opioids directly induce DNA methylation in human spermatogenesis.

4.3 STUDY INNOVATION

As opioid use remains prevalent among men of reproductive age, this study aims to observe opioid-induced alterations in the sperm methylome by utilizing a novel *in vitro* human spermatogenesis model. By generating haploid round spermatids from the differentiation of human iESCs as previously described[13,14,164,185], this research allows for the direct examination of opioid use on spermatogenesis and the developing male gametes. Given that haploid round spermatids contain all the genetic information necessary for fertilization, they provide a window into the examination of the sperm epigenome at more differentiated stage with a haploid genome. Using a combination of this model alongside high-fidelity methylation tools such as pyrosequencing, the assessment of DNA methylation patterns and levels can be assessed after exposure to clinical doses of opioids. Our focus remains on the highly relevant opioid receptor, *OPRM1*, particularly within the promoter region critical to the functionality of the gene. Although *OPRM1* has been shown to contribute to numerous health deficits in the nervous system in part due to hypermethylation after opioid exposure, the role of *OPRM1* has yet to fully be elucidated in gametes. This research provides evidence to whether opioid use can induce DNA methylation in *OPRM1* in the male gametes in our *in vitro* human spermatogenesis model, and if so, what implications these epigenetic modulations pose for these developing cells critical for reproductive success and fertilization.

While Chapter 3 focused on the impacts of exogenous opioids on Sertoli cells and the blood-testis barrier, here the attention is drawn to the cells critical for fertilization. Given the rate of opioid use among men of reproductive age, and evidence that opioids have harmful effects on the male reproductive system, particularly by disturbing hormone regulation and disrupting the HPG axis leading to hypogonadism, as well as little but existing evidence of inducing DNA methylation changes in sperm, there was a need for a novel way to assess this. The *in vitro* human spermatogenesis model by Easley and colleagues, as previously described, provides a unique and robust way to observe toxicological effects directly on developing human sperm, isolating out additional factors that are often associated with real world exposures, namely substance abuse. This direct assessment not only lets us use a variety of genetic backgrounds with variable iESCs but is also an efficient and reproducible model that is highly relevant to human exposures in the real world, thus allowing for the potential discovery of both exposure outcomes and the mechanisms involved.

4.4 RESULTS: *IN VITRO* HUMAN SPERMATOGENESIS MODEL, EPIGENETIC APPROACH, AND PRIMER DESIGN

To determine the epigenetic effects of opioids on human spermatogenesis and male gametes, an *in vitro* human spermatogenesis model as previously described was used to mimic *in vivo* human spermatogenesis and real world exogenous opioid use. Using a combination of three genetically distinct male human embryonic stem cell lines (ESCs) differentiation into spermatogenic lineages was performed as previously described[13,14,18,164,185]. Briefly, differentiating cells were maintained on mouse STO-feeder cells and maintained in spermatogonial stem cell (SSC) medium for 10 days. Clinically relevant doses of fentanyl present in serum concentrations in men were used to construct a dosing scale backed by pharmacokinetic data. Differentiating spermatogonial stem cell-like cells cultures were treated with fentanyl at concentrations of 2ng/mL, 20ng/mL, or 200ng/mL or control (0 ng/mL fentanyl). Cells were maintained in SSC media with fentanyl or medium-only controls, with medium changes occurring every other day, and a 1:1,000 dilution of bFGF and GDNF beginning on Day 1 of the differentiation to mimic long-term usage.

On Day 10 of differentiation, the cells were collected and prepared for isolation of the haploid cells specifically via fluorescence-activated cell sorting (FACS). The nuclei of live cells were stained using RedDot™1 Far-Red Nuclear Stain in order to observe the presence of a haploid peak. The DNA from the sorted cells were then isolated and prepared for DNA methylation analysis. DNA was extracted and quantified, then stored at -20°C until bisulfite conversion. To observe changes in *OPRM1*, a known target for opioids and responsible for the transcription of the mu opioid receptor, pyrosequencing primers were designed to cover a wide array of CpG sites near the promoter region of *OPRM1* (Figure 4.1A, Table 4.1A). Of the 10 primer sets created, 8 were selected after validation and used for subsequent pyrosequencing analysis post bisulfite conversion to determine DNA methylation changes at specific CpG sites along the *OPRM1* promoter region.

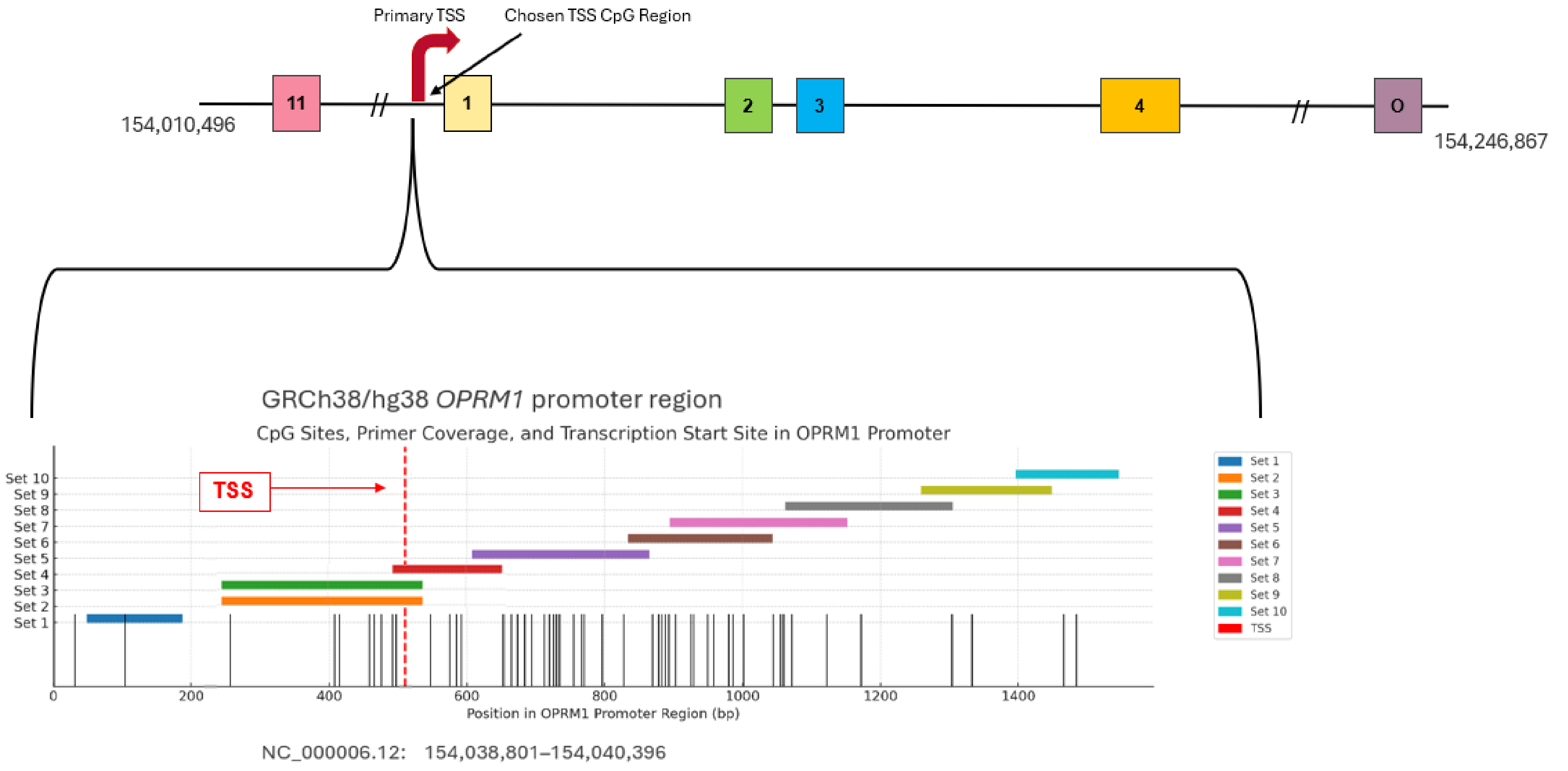
Primary TSS
Chosen TSS CpG Region
154,010,496
11
//
1
2
3
4
//
O
154,246,867
GRCh38/hg38 *OPRM1* promoter region
CpG Sites, Primer Coverage, and Transcription Start Site in OPRM1 Promoter
TSS
Set 10
Set 9
Set 8
Set 7
Set 6
Set 5
Set 4
Set 3
Set 2
Set 1
0
200
400
600
800
1000
1200
1400
Position in OPRM1 Promoter Region (bp)
Set 1
Set 2
Set 3
Set 4
Set 5
Set 6
Set 7
Set 8
Set 9
Set 10
TSS
NC_000006.12: 154,038,801–154,040,396

Figure 4.1. CpG Site distribution and primer coverage across the *OPRM1* promoter region. Distribution of CpG dinucleotides (black vertical ticks) and the coverage of ten designed primer sets (colored horizontal bars) within a ~1.6 kb region of the human *OPRM1* promoter. Coordinates are based on the GRCh38/hg38 human genome assembly, specifically chromosome 6 (NC_000006.12:154,038,801–154,040,396). The transcription start site (TSS) is indicated by a vertical dashed red line at position 224 bp. Primer sets (Set 1–Set 10) were designed to span this region to enable site-specific DNA methylation profiling. Dense CpG clustering is observed near the TSS, reflecting a potential regulatory hotspot relevant to *OPRM1* transcriptional activity.

Primer Set (in order of CpG site)	**Forward Primer (F)**	**Reverse Primer (R)**	**Sequencing Primer (S)**	**Amp. Length (bp)**
1	GGTTTGTAGGGAAGAAAATTGGAGAAATAT	TACTCTTCCATCCCTACTACCAATTTTAAT	GAAGAAAATTGGAGAAATATTATTA	138
2	GAGAAAAAGGAGTTGGAAAATTGAGTGA	CCCACCTTAATAATTCACAAAAACTCAT	TGTTTGTATAGAAGAGTGT	290
3	GAGAAAAAGGAGTTGGAAAATTGAGTGAT	CCCACCTTAATAATTCACAAAAACTCAT	CACAAAAACTCATCACCTA	290
4	GTGAGGGGTAGGTGATGAGTT	CTACCACAACTCCTACTATTTCTTAC	ATTAAGGTGGGAGGG	157
5	TAGTTAGGATTGGTTTTGTAAGAAATAG	ATTACCATCTAAATAAAACAAATTAACCC	TTTTTGTAAGAAATAGTAGGAG	257
6	TTGGGTTAATTTGTTTTATTTAGATGGTA	TTCCTTACCTAACAATCACATACA	TTAATTTGTTTTATTTAGATGGTAA	209
7	GATTTGGGAGGGAGAGATAGTTT	AACCCTTTCCTTACCTAACAATCACATAC	GGGAGGGAGAGATAGTTTG	257
8	GAGGGTTTAGAGGTTTAAGGGGGTAT	CCCCCCACCCCTAAACATTTATA	ATTTAATTTTTAAGGTTTAATGTTG	243
9	GAGAAGTTGTTTTGGTAAAAGTTATAAATG	AAAACTAACTAAAATTTCTCTCCCATTAT	GGTAAAAGTTATAAATGTTTAGGG	189
10	GTTATGGAAAGAGTAAGTTGTGAATAATG	ATAAAAAAATTACCAAAATCCATCTCAA	AGTTAGTTTTTTAAATTTGGTT	149

Table 4.1. Primer set design targeting Human *OPRM1* promoter region (GRCh38, Chr6:154,038,801–154,040,396). A summary of the primer sets designed to amplify tiled regions across the *OPRM1* promoter, based on the GRCh38/hg38 human genome assembly. Included are forward and reverse primer sequences, sequencing primers, and the amplicon size in base pairs (bp) corresponding to chromosome 6 (NC_000006.12). These primers were designed to facilitate bisulfite conversion-based DNA methylation analysis across regulatory regions upstream and near the *OPRM1* gene promoter.

4.4 RESULTS: FENTANYL INDUCED DNA METHYLATION CHANGES IN THE PROMOTER OF *OPRM1* IN HUMAN *IN VITRO* MALE GAMETES

To determine the epigenetic effects of opioids on human spermatogenesis and male gametes, we differentiated three genetically distinct iESCs into round haploid spermatids while exposing them to clinically relevant serum levels of the opioid fentanyl at doses 0 ng/mL (control), 2 ng/mL, 20 ng/mL, and 200 ng/mL over the course of 10 days, then used FACS to sort our cell population of interest and isolated out the DNA. To determine if this opioid had the ability to induce epigenetic changes in our human male-derived germ cells, we utilized a targeted approach to specifically look for changes in *OPRM1*. Being the critical gene responsible for the transcription of the mu opioid receptor, and with some literature suggesting not only its importance in spermatogenesis and male reproductive health but also noting its epigenetic vulnerability to opioid exposure. To both validate and observe specific changes in methylation in our human *in vitro* model, pyrosequencing primers were designed to cover a wide array of CpG sites near the promoter region of *OPRM1*.

Post DNA collection, primer design, and bisulfite conversion, pyrosequencing alongside statistical analyses were conducted to observe methylation changes and to focus on our targeted gene of interest. Due to a lack of DNA content post-sorting for each of the genetically distinct iESC lines, the following results represent the combined pool of the iESC line methylation data. DNA methylation changes in the CpG sites of the *OPRM1* promoter region were assessed as well as CpG sites that exhibited significant changes were identified. (Figure 4.2 A; Table 4.2 A) Overall levels of methylation change as well as volcano plots showing the most changed CpG sites for each dose (Figure 4.3 A-C) and fold changes in specific CpG sites within that region are depicted (Figure 4.4). In summary, significant dose-dependent changes in DNA methylation were observed in 18 CpG sites within the *OPRM1* promoter region, with noteworthy CpG sites near key functional regions of the gene such as the transcription start site (TSS) and transcription factor (TF) binding sites having increased DNA methylation levels corresponding to opioid use. To further investigate how DNA methylation changes in this region may impact *OPRM1* function and thus spermatids, both JASPAR predictions and ENCODE ChIP-seq data were assessed to determine TF binding sites and potential methylation-sensitive binding disruptions.

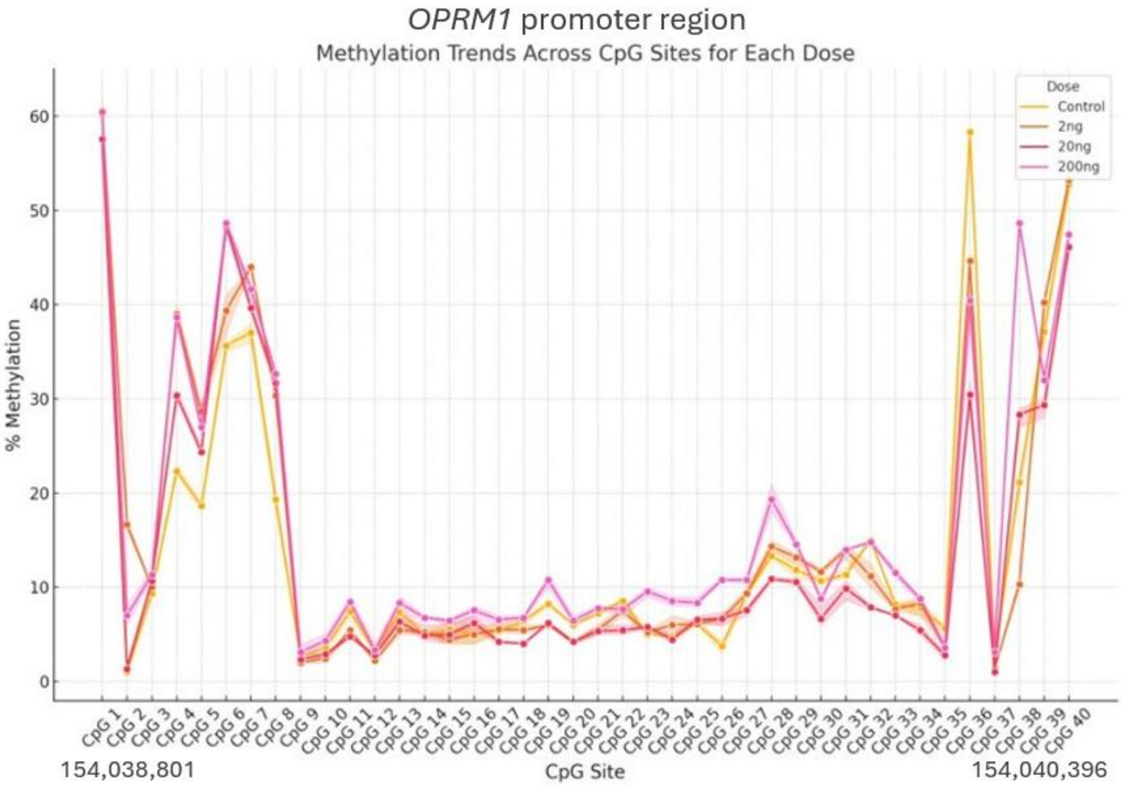
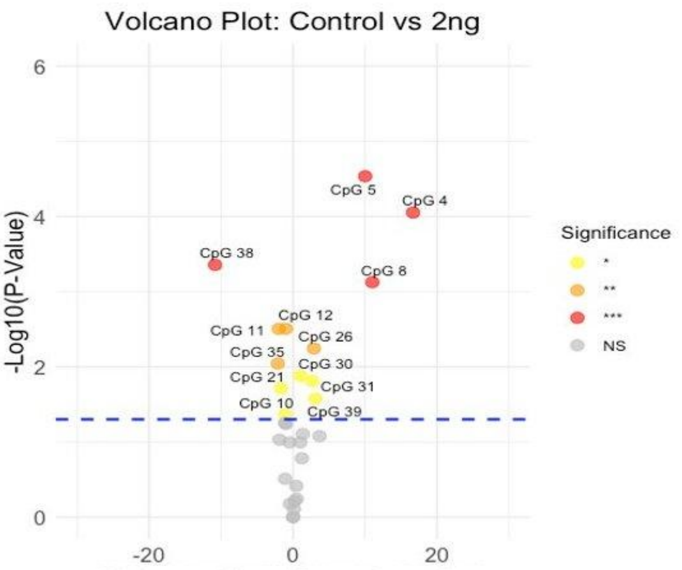

OPRM1 promoter region
Methylation Trends Across CpG Sites for Each Dose
Dose
Control
2ng
20ng
200ng
% Methylation
0
10
20
30
40
50
60
CpG 1
CpG 2
CpG 3
CpG 4
CpG 5
CpG 6
CpG 7
CpG 8
CpG 9
CpG 10
CpG 11
CpG 12
CpG 13
CpG 14
CpG 15
CpG 16
CpG 17
CpG 18
CpG 19
CpG 20
CpG 21
CpG 22
CpG 23
CpG 24
CpG 25
CpG 26
CpG 27
CpG 28
CpG 29
CpG 30
CpG 31
CpG 32
CpG 33
CpG 34
CpG 35
CpG 36
CpG 37
CpG 38
CpG 39
CpG 40
154,038,801
CpG Site
154,040,396

Figure 4.2. Opioid-induced DNA methylation trends across CpG sites in *OPRM1* promoter. Illustrated representation of the average DNA methylation levels (% methylation) across 40 individual CpG sites within the *OPRM1* promoter region following exposure to escalating concentrations of fentanyl (2 ng/mL, 20 ng/mL, 200 ng/mL), compared to untreated controls in the haploid round spermatids from our human *in vitro* spermatogenesis model. Each point represents the mean methylation percentage at a given CpG site, with lines connecting sites in sequential genomic order. Color-coded lines correspond to each dose group, revealing patterns of site-specific and dose-dependent methylation. CpG sites such as CpG 2–8 and CpG 36–40 exhibit strong methylation shifts in response to higher fentanyl doses, suggesting region-specific sensitivity to opioid exposure, highlighting the dynamic epigenetic response of *OPRM1* to opioids in a dose-dependent manner.

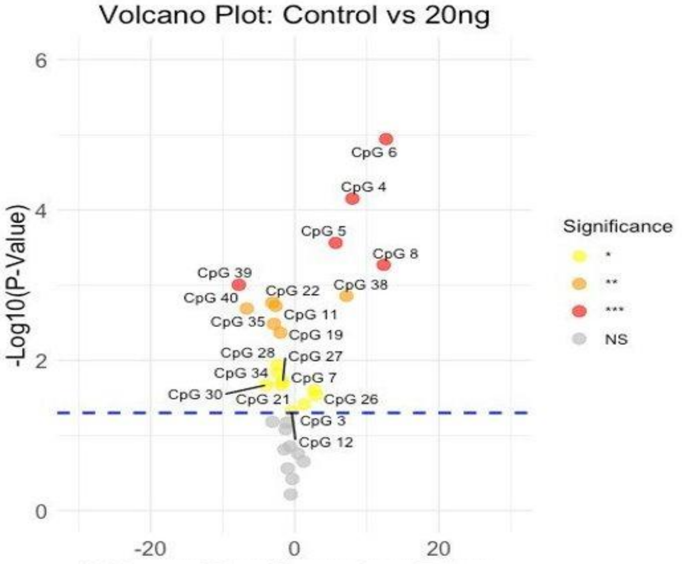
CpG Site	**Distance from TSS (bp)**	**Distance from ATG (bp)**	**Significance (p – value)**	**R² Value**
3	-80	-174	$p < 0.01$	0.816
4	-71	-122	$p < 0.001$	0.429
5	-60	-115	$p < 0.001$	0.363
6	-50	-72	$p < 0.001$	0.877
8	-32	-54	$p < 0.001$	0.734
14	-27	-49	$p < 0.01$	0.417
16	84	124	$p < 0.05$	0.568
23	159	196	$p < 0.001$	0.732
24	186	200	$p < 0.001$	0.417
25	250	204	$p < 0.001$	0.678
26	271	225	$p < 0.001$	0.837
30	313	297	$p < 0.001$	0.316
33	329	353	$p < 0.001$	0.363
35	409	363	$p < 0.001$	0.492
36	418	372	$p < 0.001$	0.567
38	445	399	$p < 0.001$	0.643
39	465	419	$p < 0.01$	0.439
40	479	923	$p < 0.001$	0.664

Table 4.2. Genomic positioning and regression strength of significantly methylated CpG sites in the *OPRM1* promoter. This table highlights individual CpG sites profiled within the *OPRM1* promoter, including their distance (bp) from the annotated transcription start site (TSS) and translation start site (ATG) based on the GRCh38/hg38 human genome assembly (Chr6:154,038,801–154,040,396) with negative values indicating upstream locations. For each site, the $\log_2$ fold change in methylation relative to control is shown for significant comparisons, along with corresponding p-values or statistical significance levels (e.g., * (0.05), ** (0.01), *** (0.001)). CpG sites exhibiting statistically significant differential methylation (adjusted $p\text{-value} < 0.05$) are annotated accordingly.

A


Volcano Plot: Control vs 2ng
-Log10(P-Value)
6
4
2
0
-20
0
20
CpG 5
CpG 4
CpG 38
CpG 8
CpG 12
CpG 11
CpG 26
CpG 35
CpG 30
CpG 21
CpG 31
CpG 10
CpG 39
Significance
*
**
***
NS

B


Volcano Plot: Control vs 20ng
-Log10(P-Value)
6
4
2
0
-20
0
20
CpG 6
CpG 4
CpG 5
CpG 8
CpG 39
CpG 38
CpG 40
CpG 22
CpG 35
CpG 11
CpG 19
CpG 28
CpG 27
CpG 34
CpG 7
CpG 30
CpG 21
CpG 26
CpG 3
CpG 12
Significance
*
**
***
NS

C

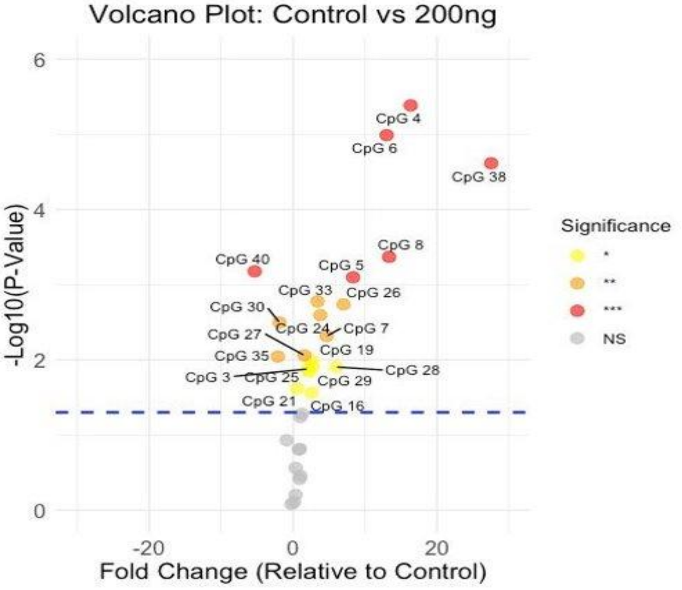

Volcano Plot: Control vs 200ng
-Log10(P-Value)
6
4
2
0
-20
0
20
Fold Change (Relative to Control)
CpG 4
CpG 6
CpG 38
CpG 8
CpG 40
CpG 5
CpG 33
CpG 26
CpG 30
CpG 24
CpG 7
CpG 27
CpG 19
CpG 35
CpG 28
CpG 3
CpG 25
CpG 29
CpG 21
CpG 16
Significance
*
**
***
NS

Figure 4.3. Volcano plots of CpG DNA methylation differences between control and fentanyl dosing groups. Volcano plot visualization site-specific changes in DNA methylation between 0 ng control samples and cells exposed to 2 ng/mL (A), 20 ng/mL (B), and 200 ng/mL (C) fentanyl across 40 CpG sites within the *OPRM1* promoter. The x-axis represents the methylation fold change relative to control, while the y-axis indicates statistical significance as $-\log_{10}$(p-value). The horizontal dashed line marks the $p = 0.05$ significance threshold. Statistical means were obtained and run in triplicate using ANOVA. Each point corresponds to an individual CpG site, with color-coded significance levels: Red (*): $p \leq 0.001$, Orange (): $p \leq 0.01$, **Yellow (*): $p \leq 0.05$, and **Gray (NS): $p > 0.05$.

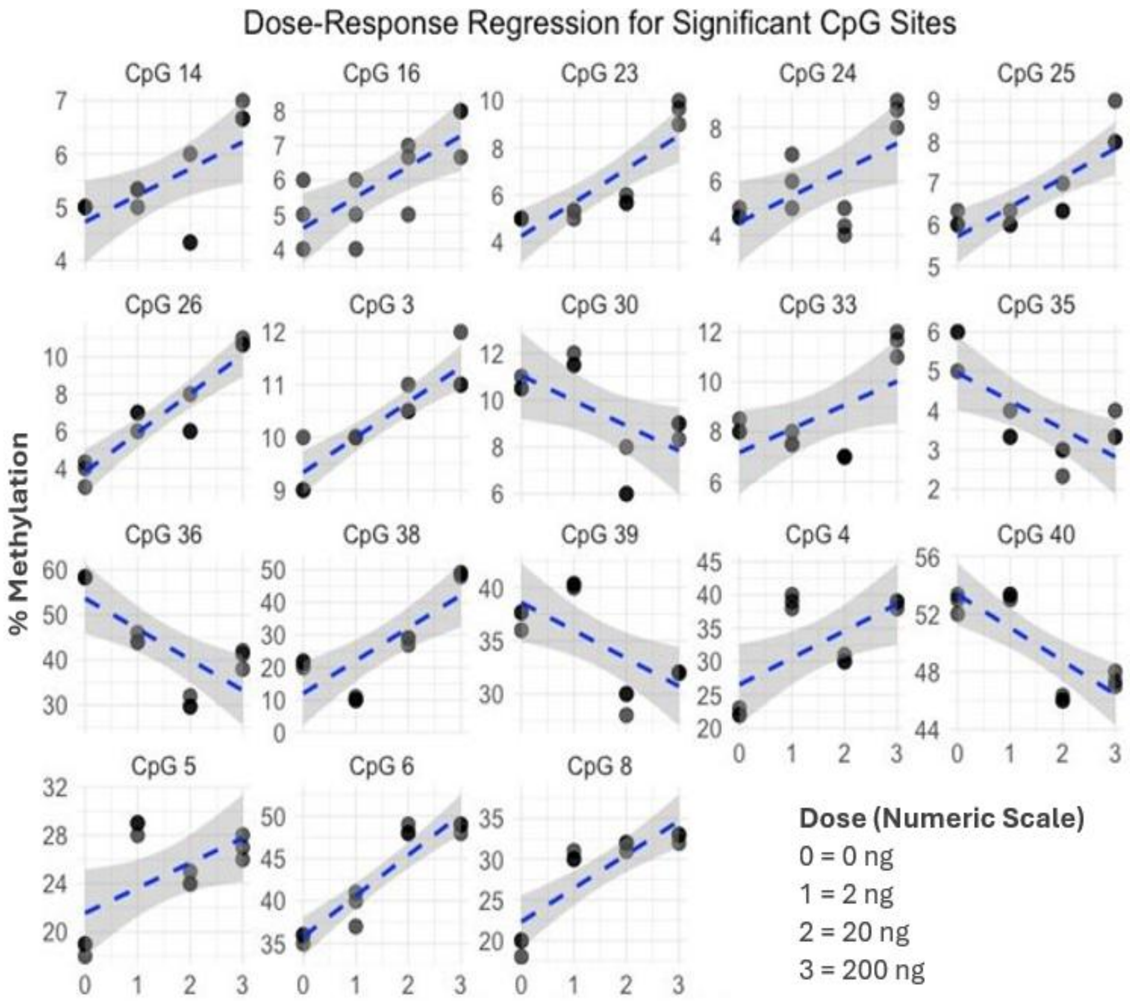

Dose-Response Regression for Significant CpG Sites
% Methylation
CpG 14
CpG 16
CpG 23
CpG 24
CpG 25
CpG 26
CpG 3
CpG 30
CpG 33
CpG 35
CpG 36
CpG 38
CpG 39
CpG 4
CpG 40
CpG 5
CpG 6
CpG 8
0
1
2
3
Dose (Numeric Scale)
0 = 0 ng
1 = 2 ng
2 = 20 ng
3 = 200 ng

Figure 4.4. Dose-response regression analysis of significant differentially methylated CpG sites. To observe trends in methylation across significant CpG sites, dose regression analysis was conducted, for each dose represented numerically (0) Control 0 ng/mL fentanyl, (1) 2 ng/mL fentanyl, (2) 20ng/mL fentanyl, and (3) 200 ng/mL fentanyl, respectively. The relative R-squared values corresponding to the CpG sites in order are as follows: CpG 14 = 0.417, CpG 16 = 0.568, CpG 23 = 0.732, CpG 24 = 0.417, CpG 25 =0.678, CpG 26 = 0.837, CpG 3 = 0.816, CpG 30 = 0.316, CpG 33 = 0.363, CpG 35 = 0.492, CpG 36 = 0.567, CpG 38 = 0.643, CpG 39 = 0.439, CpG 4 = 0.429, CpG 40 = 0.664 , CpG 5 = 0.363, CpG 6 = 0.877, CpG 8 = 0.734.

4.5 DISCUSSION

This study provides novel evidence that escalating doses of fentanyl induce dose-dependent DNA methylation changes at specific CpG sites within the promoter region of the mu opioid receptor (*OPRM1*) gene in *in vitro*-derived human spermatids. Previous studies have increased DNA methylation after exogenous opioid exposure [115,255,271]. Moreover, these results indicate a dose-response change in DNA methylation patterns corresponding with increasing levels of opioids; with a total of 18 CpG sites showing significant changes in DNA methylation due to fentanyl exposure, many of which demonstrated strong regression fits (e.g., CpG 6: $R^2 = 0.877$, CpG 26: $R^2 = 0.837$, CpG 3: $R^2 = 0.816$), supporting a robust dose-response relationship. As previously mentioned, *OPRM1* is the principal mediator of endogenous and exogenous opioid activity. Its promoter region contains a high-density CpG island proximal to the transcription start site (TSS), a feature associated with transcriptional regulation via DNA methylation[272-274]. Methylation of promoter-proximal CpG sites is known to repress transcription, either by preventing transcription factor (TF) binding or by recruiting methyl-binding proteins that alter chromatin structure[260]. In this study, several of the differentially methylated CpG sites are located near the TSS and within or adjacent to known transcription factor binding sites and known to regulate *OPRM1* expression, thus being potentially sensitive to methylation status[273,275].

Notably, while elevated DNA methylation levels were somewhat expected due to prior studies, the regression analysis also revealed lowered levels of DNA methylation at other CpG sites in a similar dose-dependent manner. While opioid use has been shown to typically increase DNA methylation, the reasoning for this dynamic change in methylation remains uncovered. It could be that opioids like fentanyl may raise methylation at certain transcriptionally active or stress-responsive sites (silencing them) and lower it at repressive or inaccessible regions (activating them), reflecting a potential dynamic regulatory balance rather than a global switch. To further understand the regulatory mechanisms controlling *OPRM1* expression in response to fentanyl exposure, we analyzed the proximal promoter region of the human *OPRM1* gene for TF binding motifs. Using TF motif prediction tools JASPAR and ENCODE ChIP-seq datasets, we identified multiple conserved binding sites located both upstream and downstream of the TSS, within approximately 500 bp on either side. These included motifs for Sp1, NF-κB, REST, YY1, and GATA-family transcription factors, many of which are known to mediate opioid response, neuronal differentiation, or chromatin remodeling.

Among the most well-characterized is Sp1, a GC-rich motif-binding TF that contributes to basal transcription of many housekeeping and inducible genes[276]. Sp1 binding sites are distributed throughout the *OPRM1* promoter, particularly near the TSS, and can be functionally inhibited by DNA methylation at adjacent CpG sites, highlighting the biological relevance of methylation changes in this domain. This ubiquitous transcription factor involved in basal promoter activity, binds GC-rich motifs between −100 and −60 bp upstream of the TSS and is considered essential for constitutive *OPRM1* expression in lymphocytes[277]. Importantly, Sp1 binding is methylation-sensitive, and several CpG sites within this region (e.g., CpGs 3–5 in our data) likely overlap or reside within proximity to Sp1 motifs. Methylation at these sites may obstruct Sp1 binding, contributing to reduced transcription in the cell types, as well as round spermatids.

In addition to Sp1, the cAMP response element-binding protein (CREB1) is implicated in opioid-induced signaling and has been shown to bind the *OPRM1* promoter, mediating transcriptional upregulation in response to cAMP and other second messengers[278,279]. CREB, a cAMP-responsive element-binding protein activated by PKA signaling, was predicted to bind between −170 and −140 bp relative to the TSS. Unfortunately, significant methylation changes were not detected in our analysis to indicate if this TF binding site could be affected, however, CREB is known to mediate opioid receptor upregulation following chronic opioid exposure in neurons and glia, and CREB binding can be inhibited by methylation of adjacent CpG sites.

We also identified a predicted NF-κB binding motif between −200 and −50 bp upstream of the TSS. NF-κB is a stress-activated transcription factor whose activity increases during inflammation, oxidative stress, and opioid exposure[280]. Its presence suggests a possible link between fentanyl-induced inflammatory signaling and *OPRM1* transcriptional regulation in Sertoli cells, specifically in the context of observed oxidative stress. YY1 was also noted as a Tf with a binding domain region between -150 to -50 bp of the promoter, and functions as a repressor and are particularly sensitive to epigenetic context, often interacting with methylated DNA to recruit chromatin-modifying complexes[277,281]. Predicted binding motifs in the promoter region also suggest roles for AP-1 (FOS/JUN), NR3C1, EGR1, and STAT3, many of which are responsive to environmental stress, inflammation, or drug exposure[282,283].

Importantly, many of these predicted TF binding sites are based off neuronal cell studies given the relevance of *OPRM1* in the brain and nervous system. Future studies need to observe if these TFs are relevant within the context of round spermatids and potentially other cells within the reproductive system. Notably, the presence of CpG sites within or near these TF binding motifs means that methylation changes observed may directly influence TF occupancy, providing a mechanistic link between fentanyl exposure, epigenetic modification, altered gene expression, and subsequently phenotypical consequences observed with opioid use.

In addition to the upstream motifs, we scanned for binding sites downstream of the TSS, particularly in intron 1 and exon 1. These include motifs for Sp1, REST, CTCF, STAT3, GATAm and NF-κB, overlapping with chromatin accessibility in ENCODE brain datasets, suggesting enhancer-like activity in these regions, although these are not high-confidence downstream binding sites. Many of these same TFs predicted here are typically shown to bind upstream promoter regions. Despite this, intragenic regulatory elements may facilitate gene expression even in the presence of promoter-proximal methylation and are important candidates for further functional validation.

Another possibility is that fentanyl alters the activity of DNA methyltransferases (DNMTs), which are known to respond to oxidative stress and other cellular stress signals[284], conditions inducible by opioid exposure. DNMTs are a family of enzymes responsible for catalyzing the transfer of a methyl group to cytosine residues in DNA, most commonly at CpG dinucleotides, and are a key epigenetic modification that regulates gene expression, genomic stability, and cellular differentiation[285]. Further research is required to determine the role of DNMTs are relevant within the scope of opioid-induced DNA methylation.

Together, these findings underscore the complexity of *OPRM1* regulation, involving both proximal promoter elements and downstream enhancer regions, many of which are susceptible to modulation by DNA methylation, transcriptional signaling, and chromatin context. The specific mechanisms by which fentanyl induces targeted methylation changes in the *OPRM1* promoter remain to be elucidated. Alternatively, fentanyl may interfere with chromatin remodeling or transcription factor occupancy at regulatory regions, thereby modifying local epigenetic landscapes.

Interestingly, despite identical fentanyl exposure conditions, we observed divergent molecular responses in our *in vitro*-derived iESC human round spermatids and human Sertoli cells. *In vitro*–derived spermatids displayed significant increases in CpG methylation across the *OPRM1* promoter region, a modification canonically associated with transcriptional repression. In contrast, human Sertoli cells exhibited upregulation of *OPRM1* gene expression under the same exposure conditions. This discrepancy likely reflects cell-type specific differences in chromatin context, transcription factor availability, and functional roles of the *OPRM1* gene.

Germ cells, particularly post-meiotic round spermatids, undergo extensive epigenetic remodeling during spermiogenesis and are highly sensitive to environmental cues that disrupt this tightly regulated process[193,270]. There is scarce evidence of the functional roles of *OPRM1* during spermatogenesis, and even less among the specific stages of differentiating gametes. Increased DNA methylation at promoter-proximal CpG sites in spermatids could serve to prevent aberrant activation of somatic genes or signaling pathways, including those mediated by the μ-opioid receptor. The repression of *OPRM1* in spermatids may represent a protective mechanism to maintain genomic and developmental integrity. Alternatively, the observed increase in DNA methylation at the *OPRM1* promoter in round spermatids raises concerns regarding potential disruptions in germ cell development and long-term reproductive and offspring health. Developing spermatids are in a uniquely vulnerable haploid state, during which epigenetic reprogramming is crucial for proper maturation and genomic integrity. Hypermethylation of promoter regions at this stage could prematurely silence key genes, including *OPRM*, which has been suggested to play emerging roles in mitochondrial function, chromatin organization, and regulated cell death[225,286-289]. The dysregulation of these processes may lead to defective chromatin compaction, impaired acrosome formation, or abnormal mitochondrial performance later in spermiogenesis[136,290]; all of which could compromise sperm functionality or viability.

In contrast, Sertoli cells, which are terminally differentiated and mitotically quiescent, possess a more permissive chromatin environment and rely heavily on environmental sensing to maintain testicular homeostasis. In this context, fentanyl-induced upregulation of *OPRM1* may reflect an adaptive cellular response mediated by opioid-responsive transcription factors such as Sp1 or NF-κB, whose activation is known to override certain epigenetic barriers[291,292]. These factors are abundantly expressed in somatic cells and are activated by cAMP and calcium signaling cascades; pathways that are often modulated by opioid receptor ligation[293,294]. Given the dynamic and resilient nature of the BTB, there are likely mechanisms governing different mediated responses involving the endogenous opioid system and its role within the male reproductive tract that differ among the cell types that express *OPRM1*.

Furthermore, the regulatory architecture of *OPRM1* likely differs from the cells within the reproductive tract compared to other cells where it is expressed in the body. *OPRM1* is broadly expressed across the peripheral and central nervous systems, where receptor interactions between endogenous opioid peptides and the *mu* opioid receptor modulate a broad variety of physiological functions beyond reward and pain perception. *OPRM1* is tightly regulated in a cell-type specific manner, and these differences are crucial for mediating the diverse effects of opioids even among neuronal cell types[295]. While the complexity and diversity of opioid receptors within the nervous system has been investigated, little work has focused on the cell-specific expression, transcription, and functions of *OPRM1* within the cells of the male reproductive tract.

Regarding these differences in gene expression levels and DNA methylation across cell types, it is possible that Sertoli cells may utilize alternative promoters or enhancer elements located distal to the canonical TSS, allowing for transcriptional activation despite local promoter methylation. Alternatively, opioid-induced signaling may stabilize *OPRM1* mRNA post-transcriptionally, or initiate chromatin remodeling independent of DNA methylation status. Developing germ cells in spermatogenesis, however, may not have the capability to moderate opioid exposure due to their extensive remodeling throughout differentiation.

Taken together, these findings underscore the importance of cellular context in interpreting the molecular consequences of opioid exposure. While the exact reasoning behind the observed increased DNA methylation levels in germ cells and, conversely, increased *OPRM1* gene expression in Sertoli cells are unknown, it could be speculated that these somatic support cells may leverage these receptors to modulate cellular stress responses and barrier function under toxicant challenge, while the developing germ cells may be susceptible to these alterations given there vulnerable nature during spermatogenesis.

These results align with prior reports showing altered *OPRM1* methylation levels in response to opioid exposure [115,198,268,271,296]. While most studies have observed opioid-induced DNA methylation changes, typically within the brain, few have looked at the possibility of negative outcomes arising from it. A notable study found that prenatal opioid exposure has been associated with altered *OPRM1* methylation in cord blood, correlating with neonatal abstinence syndrome severity[92], giving rise to the possibility of altered epigenetic landscapes prior to birth. However, few studies have explored *OPRM1* DNA methylation in the human male germline, and the findings present in this study fill a critical gap by demonstrating that opioid exposure could induce direct epigenetic modifications in *in vitro* derived human spermatids implicit in epigenetic inheritance and originating from paternal preconception exposure. While it remains unknown whether these epigenetic alterations in DNA methylation have the potential to be inherited, the altered methylation state of *OPRM1* alone warrants further investigation given the newfound involvement of the endogenous opioid system within the male reproductive tract.

Research has speculated on the role of endogenous opioids and their corresponding receptors within the male germline[123,125,135,136,194], and while the full involvement of opioids remains uncovered, studies have shed some light on potential functions in spermatogenesis. Research has revealed the presence of opioid receptors across different parts of the head, in the middle region, and in the tail of mature sperm in mammalian species[135,290,297]. Importantly, these findings suggest that the motility of spermatozoa is hindered via opioid exposure. Specifically, progressive motility, an important parameter to evaluate male fertility, was found to be significantly reduced in spermatozoa after incubation with the mu-receptor agonist morphine[135]. Notably, the spermatozoa used in those studies were collected from healthy donor semen samples and exposed to opioids post-ejaculation, while the findings present in this dissertation were derived from iESCs and underwent opioid exposure throughout their differentiation in our *in vitro* spermatogenesis model.

However, taking these findings into account may provide one mechanism for opioid-induced hypogonadism and thus infertility among opioid users. Semen quality, namely sperm motility, are critical factors in determining male reproductive health and success, and disruption to tail formation and function have been implicated in male-factor infertility[1,15,259,298]. Clinical observations note that asthenozoospermia is a common reproductive abnormality in individuals with opioid dependence. Given *OPRM1* has been found to play a role in motility and is susceptible to DNA methylation changes induced by opioid exposure, it is possible that these epigenetic changes in the *OPRM1* gene may result in impaired motility.

Consequently, the reduced semen quality and infertility observed in opioid users may result not only from disruptions to the endocrine system as previously described in the literature, but also from a direct impact of opioids on sperm cells, thus being a dual mechanism from exposure. Further adding weight to this concept, a prior study had found seminal abnormalities in heroin users regardless of their serum gonadotropin levels are altered or within the normal range[299], eluding to the possibility of both a systemic and direct impact via opioids on male reproductive tract.

The findings presented in this chapter not only strengthen this direct effect of opioid use on the male reproductive tract but also indicate that opioid-induced alterations may occur upstream of spermiogenesis. Rather than arising solely during the final phase of sperm maturation—when round haploid spermatids undergo nuclear condensation, acrosome and flagellum formation, cytoplasmic remodeling, and mitochondrial reorganization to become motile spermatozoa—these effects appear to originate earlier in the spermatogenic process, potentially impacting meiotic or even pre-meiotic stages. While current *in vitro* spermatogenesis models have yet to fully mimic the production of spermatozoa, this model and present findings provided novel insight into the potential for exogenous opioids to directly disrupt *OPRM1* DNA methylation levels in human pre-spermatozoan round spermatids.

In total, these results may allude to the possibility of opioid-induced epigenetic changes that disrupt the health and functionality of sperm throughout development and differentiation. These findings also carry significant implications for the developmental origins of health and disease (DOHaD) and the paternal origins of health and disease (POHaD), as well as male reproductive health by giving plausibility to the observed consequences in both men and offspring due to substance use[28]. Notably, spermatids represent a crucial epigenetic reprogramming window during spermatogenesis, during which DNA methylation patterns are finalized and can be passed to offspring[300,301]. Because of this, the sperm epigenome is particularly vulnerable to environmental toxicants that may influence this remodeling process. If such changes persist through fertilization, they may influence embryonic gene expression and increase offspring susceptibility to neurodevelopmental or behavioral disorders[20].

There is emerging evidence that some genes, namely those that undergo epigenetic alterations, may exhibit resistance to complete epigenetic reprogramming, particularly in the context of environmental exposures. During embryogenesis and germ cell development, two major waves of DNA demethylation typically erase the epigenome to reset transcriptional potential[141,270,300,302]. However, specific loci, including imprinted regions and certain regulatory genes, can escape this global erasure. Studies in animal models have demonstrated that chronic opioid exposure leads to persistent changes with altered gene expression and behavioral phenotypes in offspring[200,303,304]. While other studies have noted autism spectrum disorder-like behavior in prenatal and postnatal mice[305], few have looked specifically at paternal preconception exposures. A notable study in rats found that paternal morphine exposure enhances morphine self-administration and induces region-specific neural adaptations in reward-related brain regions of male offspring[117]. Another study observed reduced social play in adolescent male progeny without affecting drug-taking behavior in juvenile males or female offspring[306]. Furthermore, researchers have found that exposure to opiates in male adolescent rats altered the pain perception in the male offspring[307], as well paternal morphine self-administration produces object recognition memory deficits in female, but not male offspring[308].

While these results from the literature give evidence to the possibility of opioid-induced inheritable changes in offspring, they may fall short due to the complexity of epigenetic mechanisms in mammalian systems. Namely, there are discrepancies between human and animal experiments regarding the epigenetic influence of substance use disorders. However, even human studies have mixed observations due to variations in metrics among cohorts, meta-analyses, and a lack of controlled *in vitro* studies. Further research is needed to fully understand the long-term stability and reversibility of these changes. These features suggest that *OPRM1* is a candidate for environmentally induced epigenetic inheritance, with potential implications for intergenerational transmission of opioid-related phenotypes.

Overall, our findings underscore the epigenetic vulnerability of the male germline to exogenous opioids and highlight *OPRM1* as a key target of opioid-induced methylation changes. In addition, this study reinforces the idea of the male reproductive tract being directly targeted by opioid exposure, and that opioid exposure may induce epigenetic alterations in key genes relevant proper sperm production prior to spermiogenesis. This data supports a growing concern that opioid use may pose a threat long-term and extends our knowledge regarding the consequences of the current global opioid crisis.

4.6 METHODS & MATERIALS

Cell culture

National Institutes of Health-approved WA01 (H1, WiCell), WA14 (H14, WiCell), and WA23 (H23, WiCell) male, human embryonic stem cell lines (ESCs) were cultured and maintained in mTeSR Plus media (STEMCELL Technologies) on Matrigel® (Corning® Life Sciences) as previously described. Briefly, the Cells were cultured in 10 cm dishes and refed with mTeSR™ Plus every other day for five to seven days. The hESCs were passaged using either Dispase in DMEM/F12 (STEMCELL Technologies) or ReLeSR™ (STEMCELL Technologies) when the cell density reached approximately 80% confluency and plated onto Matrigel® (Corning® Life Sciences) coated plates. Routine karyotyping was performed every four to six months through WiCell's karyotyping core service to ensure proper chromosomal content and lack of translocation. The Easley lab performed routine mycoplasma contamination every six months to ensure the cell lines were free from contamination.

Spermatogenic differentiation of ESCs

Direct differentiation into spermatogenic lineages was performed as previously described. Briefly, differentiating cells were maintained on mitomycin C-inactivated mouse STO-feeder cells and maintained in mouse spermatogonial stem cell (SSC) medium containing the following: MEMalpha + L-glutamine (ThermoFisher), 0.2% Bovine Serum Albumin (MilliporeSigma), 0.2 mg/mL ascorbic acid (MilliporeSigma), 0.2% Chemically Defined Lipid Mixture (Millipore Sigma), 5 μg/mL insulin (MilliporeSigma), 10 μg/mL (MilliporeSigma), 50 μM β-mercaptoethanol (MilliporeSigma), 30 nM sodium selenite (MilliporeSigma), 10 mM HEPES (Gibco), 0.5× Penicillin/Streptomycin (Gibco), 20 ng/mL glial-derived neurotrophic factor (GDNF, Peprotech), and 1 ng/mL human basic fibroblast growth factor (hbFGF, Peprotech) for 10 days. The SSC media was gassed with a blood gas mixture consisting of 5% carbon dioxide, 5% oxygen, and balanced with 90% nitrogen for 30 seconds, and inverted several times to mix following initial media preparation. Approximately 250,000 STOs cultured in Fibroblast medium were added to each dish 24 hours after passage. After switching to the SSC medium, media changes occurred every other day for 10 days.

Opioid treatment

Since the testicular concentrations of opioids in adult men are unknown, clinically relevant serum concentrations were used to construct a dosing scale with established pharmacokinetic data. The cells were treated Fentanyl Citrate (MilliporeSigma) generously provided by Dr. Franklin West, at concentrations reflecting plasma levels in healthy adult men ranging from 2 – 200ng/mL. Differentiating spermatogonial stem cell-like cells ultures were treated with fentanyl at concentrations of 2 ng/mL, 20 ng/mL, or 200 ng/mL or control 0 ng/ml. Cells were maintained in SSC media with fentanyl or water-only vehicle controls, with media changes occurring every two days, and a 1:1,000 dilution of bFGF and GDNF beginning on Day 1 of the differentiation to mimic long-term usage. Fentanyl was stored at room temperature and protected from light. On Day 10, the cells were briefly rinsed with 1X DPBS without calcium or magnesium (ThermoFisher), collected using TrypLE™ Express (ThermoFisher), and analyzed according to the protocols described below.

Cell Sorting

On Day 10 of the differentiation protocol, cells were collected using TrypLE™ Express (ThermoFisher) and were prepared to isolate haploid cells during fluorescence-activated cell sorting (FACS). The nuclei of live cells were stained using RedDot™1 Far-Red Nuclear Stain (Biotium, Inc.) according to manufacturer's instructions in SSC medium in order to observe the presence of a haploid peak. Prepared samples were run on a FACS Aria II or Diva sorter (BD Biosciences). DNA was then isolated from collected haploid cells for DNA methylation analysis.

DNA Extraction & Quantification

DNA was extracted following the manufacturer's instructions from the sorted haploid round spermatids using the DNA Blood and Tissue Extraction Kit (Qiagen) Using the NanoDrop® 2000c spectrophotometer (Thermo Scientific), DNA integrity was measured, and only 160/180 ratios >2.0 were accepted for further processing. The samples were stored at -20°C until bisulfite conversion.

Bisulfite Pyrosequencing

The column-based EZ DNA Methylation kit (Zymo Research; Irvine, CA) was used to treat gDNA with sodium bisulfite to convert all unmethylated cytosines to uracils (that ultimately appear as thymines following downstream PCR and sequencing), while all methylated cytosines remain cytosines in the sequence. This resulted in bisulfite-modified DNA (bsDNA) at a final concentration of 10–20 ng/µL. bsDNA (10–20 ng) was then used as a template for PCR amplification for bisulfite pyrosequencing. Bisulfite pyrosequencing assay design, validation, and sequencing were performed. Ten pyrosequencing primers were designed using Pyromark Q48 Design software (Qiagen). Notably, 2 of the 10 primers ended up not being used after determining their inefficiency or overlapping targets during primer validation. Primer Designs found in Table (4.1 A). Pyrosequencing was conducted on a Pyromark Q48 (Qiagen) Pyrosequencing machine.

Statistical Analysis

R-Studio with R (version 4+) was used for data analyses and modeling. Microsoft Excel was used for data organization and graphical representations. Pyromark Design Software Q48 (Qiagen) was used to design primers and sequence. JASPAR and ENCODE ChIP-seq databases were used to determine potential TF binding sites. A two-way analysis of variance (ANOVA) with Dunnett's correction for multiple comparisons was used for most assessments, alongside one-way analysis of variance (ANOVA) and Tukey's HSD, where * is $p < 0.05$, ** is $p < 0.01$, and *** is $p < 0.001$.

CHAPTER 5

CONCLUDING REMARKS

The opioid epidemic has left a devastating toll on a global scale and continues to evolve to this day. Despite the decline in prescription opioids, the use and subsequently the overdose rates have dramatically increased due to the abundance of stronger opioids such as fentanyl entering the illicit drug market. Not only has this ongoing public health crisis lead to staggering addiction and mortality rates, but it has also remained an underappreciated threat to reproductive health, particularly for men who represent a substantial proportion opioid use. While effort has been made to alleviate some of the facets of this widespread problem, little work has been done to determine what long-term effects these powerful, addictive substances have on human health.

Research has provided some insight into the possible outcomes of extended opioid use, however, even less has been done to understand the effects of these substances on reproductive health, particularly for men. New studies have given credence to paternal exposures and how they many not only influence the reproductive health of men, but also may harm fertility, and could contribute to negative outcomes in offspring and future generations. Alongside declining sperm counts, semen parameters, and fertility on a global scale, researchers and health professionals are continuing to hunt for potential origins regarding these observations.

In summary, the collection of work within this dissertation aims to address a critical gap in our understanding of how exogenous opioids affect the male reproductive system.

By leveraging novel *in vitro* human male reproductive models, this body of work offers mechanistic insight into how opioid-induced perturbations may compromise reproductive integrity, all while being functionally and biologically relevant to the current ongoing opioid epidemic.

Collectively, the findings presented demonstrate that fentanyl exposure disrupts multiple key functions of Sertoli cells, which serve as the structural and physiological cornerstone of the blood-testis barrier, as showcased in Chapter 3. Assays revealed elevated oxidative stress in the barrier's Sertoli cells, suggesting a biologically significant stress phenotype. This oxidative burden may interfere with the dynamic remodeling of junctional complexes essential for barrier maintenance and germ cell support. Supporting this, fentanyl altered the expression of tight junction-associated genes such as *CLDN1*, *CLDN3*, and *CLDN7*, as well as oxidative stress-responsive genes including *CAT*, *SOD1*, and *ATM*, indicating a transcriptional reprogramming of the blood-testis barrier in response to opioid-use. Functional measurements regarding the blood-testis barrier further supported these findings. Longitudinal transepithelial electrical resistance data indicated aberrant barrier dynamics, with fentanyl exposure leading to atypical increases in resistance across time, possibly reflecting pathological hyper-tightening rather than physiological remodeling. Despite preserved selectivity in small solute permeability (as observed in paracellular dye-flux assays), this maladaptive tightening may hinder essential processes like germ cell migration and seminiferous epithelial turnover, contributing to impaired spermatogenesis. Together, these molecular, cellular, and biophysical changes paint a comprehensive picture of BTB dysfunction under opioid influence. Furthermore, these findings may provide insight onto the mechanisms behind opioid-induced hypogonadism, a symptom that many male opioid-users experience.

In Chapter 4, the goal was to examine the possibility of opioid-induced epigenetic change, specifically in the male germline. While alterations in Sertoli cells and thus the blood-testis barrier may attribute to poor reproductive health and fertility, there have been few studies that have looked at the effects opioids have on the actual gametes. Research has shown the male reproductive system to be modulated in some part by endogenous opioids, however, there is little known about what far more potent exogenous opioids have on the process of spermatogenesis. Being both highly regulated and subsequentially vulnerable, the effects of opioids on developing male gametes warrants examination given what we know.

Here, by examining the promoter region of *OPRM1*, the gene encoding the μ-opioid receptor, DNA methylation analysis revealed a dose-dependent increase in CpG methylation following fentanyl exposure in *in vitro*–derived spermatids, specifically in the region that modulates gene expression. These results suggest that opioids not only affect Sertoli cell and blood-testis barrier function but may also impact sperm development and health through epigenetic drug-induced alterations. Importantly, the potential to leave an epigenetic signature in the germline remains unknown but given the findings in this study along with prior studies noting the plausibility, concerns are warranted. Given that *OPRM1* expression is tightly linked to opioid sensitivity and hormonal feedback loops in reproductive tissues, this methylation changes may have functional consequences for gene regulation in sperm, thus potentially impacting sperm health and even offspring phenotype. These findings align with the paternal origins of health and disease (POHaD) framework, raising the possibility that opioid exposure could exert transgenerational effects via epigenetic inheritance.

Taken together, this combined focus on cell health, barrier function, and altering germline epigenetics broadens our understanding of how opioids may compromise male reproduction and fertility not only by disrupting adult testicular architecture but also by embedding molecular alterations with the potential for intergenerational impact. Future studies should examine if Sertoli cell *OPRM1* epigenetics are altered in a similar manner to the observations in round spermatids, and what other genes may have their epigenetic landscape altered and lead to potential downstream effects. Assessment of critical gene expression levels in round haploid spermatids would allow for a robust picture of how opioids may affect their epigenetic landscape and thus transcription, health, and development. Perhaps the most important aspect to investigate would be if exogenous opioid-induced DNA methylation of *OPRM1*, as well as other relevant genes, persist into spermiogenesis, impact fertilization, and potentially influence reproductive outcomes or developmental phenotypes in offspring.

Overall, this dissertation provides novel evidence that fentanyl compromises human male reproductive health through direct effects on Sertoli cells, blood-testis barrier integrity, and alters the epigenetic landscape of developing germ cells. These findings emphasize the importance of evaluating reproductive toxicity, especially regarding pharmaceuticals that were once renowned for their miraculous efficiency. Critical, robust strategies that account for fertility and transgenerational health risks are desperately needed overall, as well as any knowledge that can be used to mitigate further damage the opioid crisis may bring.

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APPENDIX

RT-qPCR GENE EXPRESSION PRIMERS AND NATURE UROLOGY REVIEW PAPER[1]

Table A.1. RT-qPCR Gene Expression Analysis Primers. 13 Integrated DNA Technologies (IDT) primers were designed and used along with one BIORAD PrimePCR primer, which contained forward and reverse sequences proprietary to the manufacturer. Catalog number was provided in place of forward and reverse sequences.

Gene	Primer (F or R)	Sequence (5' - 3')	Tm
GAPDH	Forward	GAC ATC AAG AAG GTG GTG AAG C	56
GAPDH	Reverse	GTC CAC CAC CCT GTT GCT GTA G	60
GATA4	Forward	CGA CAC CCC AAT CTC GAT ATG	55.1
GATA4	Reverse	GTT GCA CAG ATA GTG ACC CTG	57.2
CLDN1	Forward	CCT CCT GGG AGT GAT AGC AAG T	55.4
CLDN1	Reverse	GGC AAC TAA AAT AGC CAG ACC T	55.1
CLDN3	Forward	ACC ACC ATT ATC CGG GAC TTC T	55.9
CLDN3	Reverse	GCG GAG TAG ACG ACC TTG G	57.6
CLDN7	Forward	AGC TGC AAA ATG TAC GAC TGC	55.6
CLDN7	Reverse	TCG ACG TTT TAC ATG CTG ACG	57.3
AR	Forward	GAC GAC CAG ATG GCT GTC ATT	57.3
AR	Reverse	GGG CGA AGT AGA GCA TCC T	56.7
SOX9	Forward	AGC GAA CGC ACA TCA AGA C	57.7
SOX9	Reverse	CTG TAG GCG ATC TGT TGG GG	55.9
ATM	Forward	GAG TGC AGT GAC AGT GAT GTG T	57.6
ATM	Reverse	CAT GTT CTA GTT GAC GGC AGC A	57.3
XRCC1	Forward	CTG AAG AGA CCA AAGCAG CCT C	57.9
XRCC1	Reverse	CCC ATT GTC CTG TCC TTC TGA C	57.5
CASP9	Forward	GGT GGG GAG CAG AAA GAC C	58.1
CASP9	Reverse	AGC TGG TCG AAG GTC AA T	58.3
SOD1	Forward	AGC GAG TTA TGG CGA CGA CGA AG	57.4
SOD1	Reverse	CAA GTC TCC AAC ATG CCT CTC T	56.7
ATR	Forward	GGG CTC CCT TCT CAG TCA TGT A	58.2
ATR	Reverse	AAT CTT GCA CAG CAT CCC TGT T	57.4
CAT	Forward	ACT GTT GCT GGA GAA TCG GG	57.4
CAT	Reverse	AGG ACG TAG GCT CCA GAA GT	57.5
OPRM1	F&W	BIORAD PrimePCR Cat# 10025636	57

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Review article

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Influence of substance use on male reproductive health and offspring outcomes

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Abstract

The prevalence of substance use globally is rising and is highest among men of reproductive age. In Africa, and South and Central America, cannabis use disorder is most prevalent and in Eastern and South-Eastern Europe, Central America, Canada and the USA, opioid use disorder predominates. Substance use might be contributing to the ongoing global decline in male fertility, and emerging evidence has linked paternal substance use with short-term and long-term adverse effects on offspring development and outcomes. This trend is concerning given that substance use is increasing, including during the COVID-19 pandemic. Preclinical studies have shown that male preconception substance use can influence offspring brain development and neurobehaviour through epigenetic mechanisms. Additionally, human studies investigating paternal health behaviours during the prenatal period suggest that paternal tobacco, opioid, cannabis and alcohol use is associated with reduced offspring mental health, in particular hyperactivity and attention-deficit hyperactivity disorder. The potential effects of paternal substance use are areas in which to focus public health efforts and health-care provider counselling of couples or individuals interested in conceiving.

Sections

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Key points

- Alcohol consumption, especially chronic and heavy intake, alters the hypothalamic–pituitary–gonadal axis, male reproductive hormones, semen parameters, testicular damage and results in decreased sexual function.
- Cannabis use adversely affects male fertility, resulting in substantial testicular atrophy, altered reproductive hormones, and changes in semen parameters.
- Opioid abuse can negatively affect male reproductive hormones and spermatogenesis, and is associated with an increased risk of reduced testicular volume and erectile dysfunction.
- Nicotine smoke is linked to impaired semen parameters and erectile dysfunction, and seems to follow a dose-dependent pattern.
- Preconception paternal alcohol intake is associated with adverse short-term and long-term offspring outcomes, including birth defects, growth abnormalities, neurodevelopmental disorders and addiction vulnerability.
- Preconception paternal cannabis use has been linked to increased rates of pregnancy loss, decreased infant birthweight and offspring behavioural problems, including poor attention.

Introduction

The prevalence of substance use is rising, especially among men of reproductive age (~18–44 years old), and has been exacerbated by the COVID-19 pandemic[1–3]. The substances that are most popularly used include alcohol, cannabis, opioids and tobacco products[4]. This trend in increasing use is concerning because men are often unaware of the potential adverse effects on their reproductive health. Existing evidence indicates that substance use can influence spermatogenesis, reproductive hormone secretion via the hypothalamic–pituitary–gonadal (HPG) axis, and sexual function[5–7] (Box 1). As infertility is associated with a large emotional and economic burden[8,9], identifying modifiable behavioural or lifestyle risk factors, such as substance use, which can affect human fertility, is a considerable clinical issue and an important public health matter.

Emerging evidence also suggests that preconception paternal drug use can have adverse consequences for offspring[10] (Box 2). Historically, the focus has mostly been on the effects of maternal substance use during pregnancy[10], whereas little is known regarding paternal contributions. The paucity of knowledge in this area is partly caused by the limitations of existing studies, such as confounding, recruitment bias and patient self-reporting, which make causal interpretations challenging. Results of studies in which the effect of preconception paternal substance use on offspring has been investigated have demonstrated that paternal exposure to alcohol, cannabis, opioids and tobacco can adversely influence offspring neurodevelopment and is associated with poor offspring mental health, in particular hyperactivity, depression, attention-deficit hyperactivity disorder (ADHD) and addiction vulnerability[10,11]. The underlying cause is not well understood, but research has shown that preconception paternal substance use might alter offspring brain and neurobehavioural development through epigenetic mechanisms[10,12–15].

A comprehensive summary of the effects of substance use (alcohol, cannabis, opioids and nicotine) on male reproductive health and the short-term and long-term outcomes for offspring to guide health-care providers is lacking. In this Review, we examine the influence of substance use on male fertility and the potential for preconception paternal use to affect the health of future offspring. The preconception period is a crucial window for targeting education and counselling on the optimization of lifestyle factors, because motivation is often strong to achieve the desired health outcomes in preparation for pregnancy. Focusing public health efforts and health-care provider counselling is important to improve patient awareness.

Male fertility

Unhealthy lifestyles, including consumption of alcohol, and use of tobacco or cannabis products, are universally recognized to negatively affect general health, but their influence on male fertility is less well known. Studies are limited, but their results have shown that the most commonly used substances, alcohol, cannabis, opioids and tobacco products, are associated with adverse reproductive health outcomes[16].

Alcohol

Research on alcohol consumption and male fertility has largely demonstrated that it can alter male reproductive hormones[5,17–19], semen parameters[5,20,21], testicular volume[22,23] and sexual function[24,25] (Table 1). Results also suggest that these changes follow an exposure–response relationship with an increased effect observed after heavy and chronic alcohol consumption[26,27]. Heavy drinking in men is often defined as ≥15 drinks per week, or binge drinking on each of ≥5 days in the past 30 days[28].

Male reproductive hormones. Alcohol consumption is reported to alter the regulation of the HPG axis and affects all three components of the axis (hypothalamus, pituitary and gonads), disrupting the production of gonadotropin-releasing hormone (GnRH), follicle-stimulating hormone (FSH), luteinizing hormone (LH) and testosterone[5,17,18,29]. Specifically, alcohol can stimulate the paraventricular nucleus of the hypothalamus to release corticotropin-releasing factor (CRF)[30].

The effect of alcohol consumption on male fertility has been examined in preclinical and human studies, with varying results: results of the preclinical studies have largely demonstrated reduced levels of LH, FSH and testosterone, but the results of human studies have been conflicting[5,17–19]. In 66 men aged 36.6 ± 5.7 years (mean ± standard deviation) who consumed a minimum of 180 ml of alcohol daily for at least 5 days per week for at least a year without polysubstance use, elevated FSH, LH and oestradiol levels were observed. Testosterone and progesterone levels were also decreased[31]. Similarly, ethanol intake of ~220 g daily for 4 weeks resulted in decreased testosterone levels and metabolism, independent of cirrhosis or nutritional factors, in 11 healthy men, 21–40 years of age[32]. Taken together, these studies suggest that regular alcohol consumption, even up to 4 weeks, can alter male reproductive hormones and testosterone metabolism, which can affect fertility.

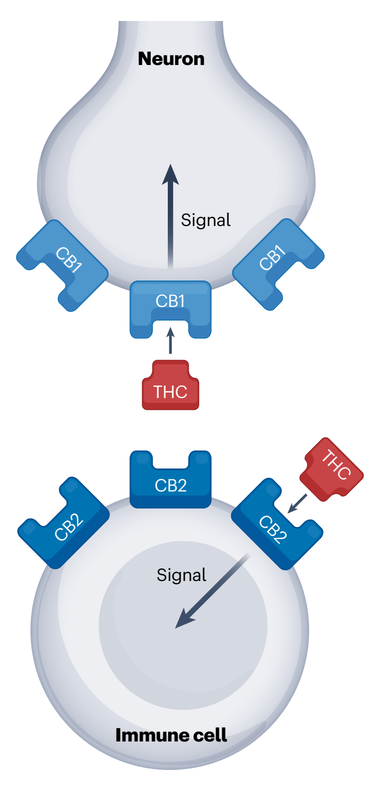
Contrary to these findings, another study of 45 men with alcoholism aged 20–40 years compared with 55 healthy men aged 22 to 32 years had significantly reduced levels of testosterone (4.96 ± 0.16 ng/ml versus 7.56 ± 0.13 ng/ml, $P < 0.001$), which correlated negatively with thiobarbituric acid reactive substances[33]. The authors hypothesized that because low testosterone was not accompanied by a rise in LH and FSH levels, but correlated with increased serum levels of oxidizing agents and decreased serum levels of reactive oxygen species,

Box 1

Summary of substance use (that is, alcohol, cannabis, opioids and nicotine) on male reproductive health

Effects of substances on male fertility can be measured using hypothalamus–pituitary–gonadal axis and semen parameter changes.

- Alcohol — ↑ luteinizing hormone (LH) and/or follicle-stimulating hormone levels and sperm DNA fragmentation; ↓ testosterone levels, seminal volume and sperm count, motility and morphology.
- Cannabis — ↓ LH and sperm count, motility and morphology; ↑ DNA fragmentation; ↓ or ↑ testosterone levels.
- Opioids — ↓ gonadotropin-releasing hormone secretion, testosterone levels and sperm motility and morphology; ↑ sperm DNA fragmentation.
- Nicotine — ↓ or ↑ LH and/or follicle-stimulating hormone; ↑ testosterone levels; ↓ sperm count, motility and morphology.



THC, Δ^9 -tetrahydrocannabinol.

these results were in part attributable to an impaired HPG axis and increased oxidative stress. In addition, a duration-dependent decrease in serum testosterone levels was observed in alcohol users, which is important for health-care providers to discuss with men, including those interested in conceiving. In 8,344 men (aged 30–80 years old) a linear association between total alcohol consumption and testosterone levels was observed, but no consistent association between alcohol consumption and serum inhibin B, FSH or LH levels was found[34]. Interestingly, young men (aged 18–28 years) and fertile men (aged 18–45 years with pregnant partners) who consumed >20 alcoholic drinks per week had higher free testosterone than men consuming 1–10 drinks weekly, probably owing to changes in testosterone metabolism by the liver[34]. This observation highlights that when testosterone is used alone as part of a fertility work-up it can be unreliable and should be combined with other reproductive health markers in alcohol users.

Research findings regarding the influence of alcohol consumption on male reproductive hormones are heterogenous, in part owing to expected intraindividual fluctuations in hormone levels and diurnal variation of sex hormones in men[35,36]. Testosterone cycles can fluctuate from increased in the morning to reduced in the evening, which can affect study results depending on the time of day that hormonal sampling is performed[35]. Thus, future studies need to harmonize timing of blood collection for male reproductive hormone assessment.

Semen parameters. Overall, animal and human studies suggest that alcohol use, especially chronic and heavy intake, is associated with reduced sperm concentration and motility[5,20,21], increased abnormal sperm morphology[5,20,21] and increased DNA damage, including defects in chromatin condensation[5,21]. In 2011, results of a meta-analysis of 57 studies including 29,914 participants aged ≥13 years showed a significant association between alcohol consumption by men and reduced semen volume (pooled mean difference (MD) −0.30, $P = 0.007$) in both healthy men and those with infertility. This meta-analysis was limited by heterogeneity between studies and inclusion of only cross-sectional studies, but it highlights modifiable lifestyle factors that can improve male fertility[37]. In another study, 66 men (aged 36.6 ± 5.7 years) undergoing care at an addiction treatment centre, who consumed a minimum of 180 ml of alcohol per day for at least 5 days a week for 1 year, were found to have significantly decreased semen volume (1.56 ± 0.79 versus 2.17 ± 0.712), sperm count (51.99 ± 44.71 versus 132.97 ± 89.02), motility (30.38 ± 15.82 versus 56.10 ± 8.72) and morphologically normal sperm (67.17 ± 16.73 versus 82.00 ± 9.76) compared with healthy men ($P < 0.001$)[31]. An autopsy series including men (aged 49–61 years) who were light drinkers ($n = 32$, daily intake <10 g) and heavy drinkers ($n = 44$, daily intake> 80 g) reported that more than half of heavy drinkers ($n = 23$) had partial or complete spermatogenic arrest ($P < 0.0001$)[38]. Similarly, in 20 men (aged 25–42 years) with alcohol dependence syndrome, a decrease in semen sample volume (0.5–2.5 ml versus 3–4.5 ml), sperm concentration (less than 50 million/ml versus 60–164 million/ml), and normal sperm motility (20–45% versus >50%) was observed compared with men without a history of alcohol consumption[39]. The authors of this study concluded that chronic alcohol consumption can influence spermatogenesis and spermiogenesis, resulting in oligozoospermia[39]. By contrast, results of an international cross-sectional study including 8,344 healthy men (aged 18–45 years old) did not show any consistent association between semen parameters and low-to-moderate alcohol consumption (median weekly intake was 8 drinks)[34].

The data are mixed, but the evidence largely supports an adverse effect of alcohol consumption on semen parameters that is probably dose dependent and time dependent. Contributing to the heterogeneity of the data is the variability in semen quality among healthy men[40] and because studies varied in the degree of confounder adjustment. Moreover, moderate-to-heavy alcohol use could affect other physiology that can influence semen parameters and might account for the mixed evidence. To improve evaluation of the consequences of alcohol consumption on semen characteristics, future research must adequately adjust for confounding variables, especially polysubstance use.

Testicular volume. Heavy alcohol use can result in testicular atrophy[22,23], especially in men with advanced alcoholic cirrhosis[25], which can be caused by direct testicular damage secondary to an adverse effect on the HPG axis, and other common confounders such as polysubstance use or malnutrition[23]. In a study including 55 men with cirrhosis of the liver, testicular atrophy was present in 70% of patients in whom the size of the testes were measured[25]. Testicular atrophy, assessed using testicular tissue obtained as soon as possible post-mortem, has been reported to be primarily a result of decreased seminiferous tubule diameter and loss of germ cells[41]. The loss of germinal tissue is thought to be secondary to primary testicular failure given the presence of elevated FSH in this study[41]. Testicular shrinkage, in part owing to the loss of germ cells, can be associated with reduced sperm count and quality, which can adversely affect male fertility[42]. Overall, the current literature suggests that alcohol consumption is associated with a dose-dependent effect on testicular volume, but more studies are needed to determine whether these changes are reversible with alcohol cessation.

Erectile dysfunction and sexual function. Evidence indicates that a relationship exists between alcohol intake and erectile dysfunction[26,43,44]. A 2018 meta-analysis of 24 studies including 154,295 patients (aged 18–79 years) reported that light-to-moderate (<21 drinks weekly) alcohol consumption was linked to a protective effect against erectile dysfunction (odds ratio (OR) 0.71, 95% CI 0.59–0.86)[26]. However, regular and heavy alcohol intake (>21 drinks weekly) had no significant effect on the risk of erectile dysfunction (OR 0.99, 95% CI 0.81–1.22, $P = 0.892$)[26]. In this study, a non-linear relationship between alcohol intake and risk of erectile dysfunction was reported, potentially because of confounding influences from lifestyle factors[26]. A major limitation of this meta-analysis was that it included only cross-sectional studies, limiting the strength of evidence of a causal relationship between alcohol and erectile dysfunction[26]. Similarly, results of a meta-analysis from 2021 including 46 studies ($n = 216{,}461$) showed a protective effect between weekly alcohol intake and erectile dysfunction (OR 0.89, 95% CI 0.81–0.97); light to moderate (≤14 drinks per week, OR 0.82, 95% CI 0.72–0.94) and high (≥14 drinks per week, OR 0.82, 95% CI 0.67–1.00)[44]. A protective association possibly exists between alcohol consumption and erectile dysfunction, but the study was limited by high heterogeneity, selection and recall biases, as well as confounding variables[44].

Increased rates of sexual dysfunction have been reported in alcohol-dependent populations compared with social drinkers or healthy individuals[24]. The most common issues reported include premature ejaculation, delayed ejaculation, decreased libido and reduced sexual potency[24,25]. In an observational study including 2,112 pregnant couples in the UK in which time to pregnancy (months) was assessed[27], paternal alcohol consumption significantly affected fecundity, especially if they consumed >20 alcoholic beverages a week (18.6, 95% CI 15.7–21.3, $P < 0.001$)[27]. This observation suggests a possible dose-dependent effect of alcohol consumption on fecundity and supports promotion of a healthy lifestyle among individuals planning or trying for pregnancy.

Box 2

Summary of paternal substance use (that is, alcohol, cannabis, opioids and nicotine) on offspring development

Effects on fetal development
- Alcohol — increased intrauterine growth restriction
- Cannabis — increased pregnancy loss
- Opioids — decreased fetal weight
- Nicotine — increased pregnancy loss

Effects on infant development (<1 year old)
- Alcohol — increased birth defects, decreased birthweight
- Cannabis — increased congenital cardiac anomalies
- Opioids — increased withdrawal-like behaviour
- Nicotine — increased testosterone levels

Effects on childhood and adolescent development (1–19 years old)
- Alcohol — increased risk of cancers (leukaemia and brain tumours), psychopathological disorders and substance-related disorders
- Cannabis — increased behavioural issues
- Opioids — increased risk of opioid addiction, delayed learning and impulsive behaviours
- Nicotine — decreased sperm count, increased risk of neurodivergent behaviour (such as autism)

Alcohol consumption is associated with erectile dysfunction and sexual function, especially in exposure-duration and dose-dependent manners. Individuals that are sexually active would benefit from cessation of or reduced alcohol consumption, especially if they have sexual dysfunction already.

Summary. In general, preclinical and human research suggests that alcohol consumption negatively influences male reproductive health, including male reproductive hormones, semen parameters, testicular volume and sexual function (Table 1). These effects seem to be linked to the duration and amount of alcohol consumed; regular or daily alcohol intake can negatively affect male fertility and make conception increasingly difficult, but occasional consumption has not been consistently shown to have an adverse effect[45]. Currently, no guidelines exist regarding male fertility and a safe or preferred amount of alcohol to consume, but, importantly, health-care providers need to screen their patients and counsel them regarding limiting the frequency and volume of alcohol exposure, and to avoid daily or binge drinking.

Cannabis

The prevalence of cannabis use is rising, especially among men of reproductive age, in part owing to growing societal acceptance and legalization[6,46]. Results from a 2021 United States national survey found that 22.1% (~27.2 million) men aged 18 years or older reported cannabis

Table 1 | Effect of alcohol on reproductive health and offspring outcomes

Year	Primary measure	Species	Study design	Conditions	Results	Ref.
Reproductive health						
1992	Male reproductive hormones	Rat ($n=24$ total, 12 ethanol-treated and 12 control)	Experimental study	6% ethanol diet for 5 weeks	Ethanol exposure was associated with significantly lower serum and testicular testosterone than untreated animals ($P<0.01$)	17
1977	Male reproductive hormones	Mouse ($n=28$ total, 10 in control, 8 in ethanol-treated and 10 in sucrose-fed controls)	Experimental study	Intragastric administration of ethyl alcohol (1.24 g/kg)	Ethyl alcohol was associated with a transient decrease in plasma testosterone that recovered after 1 h	19
2011	Semen parameters	Human ($n=209$)	Retrospective study	Self-reported alcohol consumption	Heavy alcohol use (>10 drinks/week) was significantly associated with reduced sperm count ($P=0.04$). No significant association between level of alcohol use and the high DNA stainability or DNA fragmentation index of sperm	21
2018	Erectile dysfunction and sexual function	Human ($n=154{,}295$)	Meta-analysis	Self-reported alcohol consumption	Light-to-moderate alcohol consumption (<21 drinks/week) correlated with a decreased risk of erectile dysfunction (OR 0.71, 95% CI 0.59–0.86, $P=0.000$). Regular and heavy alcohol consumption (>21 drinks/week) had no significant effect on the prevalence of erectile dysfunction. A non-linear relationship was observed between alcohol consumption and risk of erectile dysfunction	26
2004	Sexual function	Human ($n=2{,}112$)	Observational study	Self-reported alcohol consumption	Paternal consumption of >20 drinks/week before conception was associated with a significantly increased time to pregnancy ($P<0.001$)	27
2023	Male reproductive hormones	Human ($n=23{,}258$)	Meta-analysis study	Self-reported alcohol consumption	Alcohol consumption significantly decreased semen volume (SMD −0.51, 95% CI −0.77 to −0.25), but was not significantly associated with changes in sperm density, mobility and morphology Alcohol consumption was linked to decreased testosterone levels (SMD −1.60, 95% CI −2.05 to −1.15), follicle-stimulating hormone (SMD −0.47, 95% CI −0.88 to −0.05), luteinizing hormone (SMD −1.35, 95% CI −1.86 to −0.83), but no effect on oestradiol, inhibin B and sex hormone-binding globulin	29
2005	Male reproductive hormones and semen parameters	Human ($n=66$)	Observational study	Self-reported alcohol consumption	Alcohol consumption was associated with significantly ($P<0.001$) increased follicle-stimulating hormone, luteinizing hormone, oestradiol levels, semen volume, sperm count, sperm motility and morphologically normal sperm, in addition to decreased testosterone and progesterone levels	31
2006	Male reproductive hormones	Human ($n=46$)	Observational study	Self-reported alcohol consumption	Alcohol consumption was associated with significantly ($P<0.001$) low plasma testosterone, luteinizing hormone and follicle-stimulating hormone	33
2014	Semen parameters	Human ($n=8{,}344$)	Cross-sectional study	Self-reported alcohol consumption	No consistent association between semen parameters and alcohol consumption. A positive linear association between total alcohol consumption and total or free testosterone was observed	34
2011	Semen parameters	Human ($n=29, 914$)	Systematic review and meta-analysis	Self-reported alcohol consumption	Alcohol consumption is a risk factor for reduced semen volume and quality	37
1994	Semen parameters	Human ($n=44$)	Observational study	Family-reported alcohol consumption	Heavy alcohol consumption (>80 g per day) was associated with an increased risk of partial or complete spermatogenic arrest ($P<0.001$) and Sertoli cell-only syndrome ($P<0.05$)	38
1985	Semen parameters and male reproductive hormones	Human ($n=20$)	Experimental study	Alcohol dependence syndrome	Alcohol dependence syndrome was associated with a decrease in total semen volume, sperm concentration and testosterone, in addition to an increased percentage of morphologically abnormal sperm. No association of alcohol dependence syndrome and changes in luteinizing hormone, follicle-stimulating hormone and prolactin were observed	39
2021	Erectile dysfunction and sexual function	Human ($n=216{,}461$)	Meta-analysis	Self-reported alcohol consumption	Alcohol consumption was associated with erectile dysfunction (OR 0.89, 95% CI 0.81–0.97); light to moderate consumption (OR 0.82, 95% CI 0.72–0.94); heavy consumption (OR 0.82, 95% CI 0.67–1.00)	44

Table 1 (continued) | Effect of alcohol on reproductive health and offspring outcomes

Year	Primary measure	Species	Study design	Conditions	Results	Ref.
Reproductive health (continued)						
2017	Semen parameters	Human (*n*=16,395)	Systematic review and meta-analysis	Self-reported alcohol consumption	Alcohol consumption adversely affected semen volume and sperm morphology	45
2013	Sperm parameters	Mouse	Experimental study	5% or 10% ethanol exposure	Paternal ethanol consumption was associated with decreased sperm count and motility, in addition to increased abnormal sperm morphology, apoptosis, DNA integrity and chromatin remodelling	182
2018	Sperm parameters	Mouse (*n*=18, 9 ethanol-treated and 9 control)	Observational study	Chronic ethanol consumption (vapour ethanol for 8 h per day, 5 days a week, for 5 weeks)	Paternal chronic ethanol exposure was associated with altered sperm small non-coding RNAs and reduced epididymal expression of a tRNA-derived small RNA methyltransferase, *Nsun2*, known to directly regulate tRNA-derived small RNA biogenesis	183
Offspring outcomes						
2019	Short-term and long-term offspring outcomes	Mouse (*n*=11 total, 6 alcohol-exposed and 5 control)	Experimental study	4-h nightly exposure to 10% ethanol solution	Paternal alcohol exposure before conception resulted in decreased offspring weight and increased insulin hypersensitivity in male offspring	174
2023	Short-term offspring outcomes	Mouse (*n*=192 total, 96 alcohol-exposed and 96 control)	Experimental study	Weight-based alcohol dosing (2.7 g/kg)	Paternal alcohol exposure before conception was associated with offspring craniofacial abnormalities and growth deficiencies that are dose dependent	175
2021	Short-term offspring outcomes	Human (*n*=529,090)	Prospective, population-based study	Self-reported alcohol consumption	Paternal alcohol consumption before conception was associated with an increased risk of birth defects (OR 1.35, 95% CI 1.14–1.59; $P<0.001$)	176
2014	Short-term and long-term offspring outcomes	Mouse (*n*=123, 45 3.3 g/kg ethanol exposed, 39 1.1 g/kg ethanol exposed, and 39 control)	Experimental study	Intragastric ethanol exposure (0, 1.1, 3.3 g/kg)	Chronic paternal ethanol exposure (1.1 or 3.3 g/kg) before conception altered methylation of imprinted genes in sire sperm and in the offspring's cerebral cortices that are linked to delayed offspring cognitive performance, and increased anxiety and depression	177
2007	Short-term and long-term offspring outcomes	Mouse (*n*=45 total, 15 males, and 30 females). Single-case experimental design, same 15 males were exposed to saline and then to ethanol	Experimental study	Acute alcohol exposure (single dose 5 g/kg of 20% ethanol)	A single acute paternal alcohol exposure before insemination was linked to early offspring developmental delays and increased aggression	178
2018	Short-term offspring outcomes	Human (*n*=1,292)	Observational study	Self-reported alcohol consumption	Paternal alcohol consumption (amount not defined) 3 months before conception was associated with a shorter anogenital distance, a biomarker of reproductive hormone abnormalities, in offspring, especially male offspring	179
1994	Short-term offspring outcomes	Rats (*n*=80 total, 20 male controls, 20 males at 1.25 g/kg, 20 males at 2.5 g/kg, and 20 males at 5 g/kg)	Observational study	Single acute alcohol exposure (saline, 1.25 g/kg, 2.5 g/kg, or 5 g/kg of 20% ethanol)	Paternal alcohol administration 24 h before breeding decreased the number of viable offspring and increased offspring mortality	180
2009	Long-term offspring outcomes	Human (*n*=1,252)	Observational study	Self-reported alcohol consumption	Paternal alcohol dependence was associated with an increased risk of externalizing disorders and psychopathology in late adolescence in children	185
2020	Short-term and long-term offspring outcomes	Human (*n*=4,726)	Observational study	Self-reported alcohol consumption	Paternal alcohol consumption (at least 50 ml per day) 3 months before conception was associated with an increased risk of offspring congenital heart disease (aOR 2.87, 95% CI 2.25–3.65)	186
2022	Long-term offspring outcomes	Human (*n*=64,710)	Observational study	Self-reported alcohol consumption	Paternal alcohol consumption (20–100 g per week) was associated with an increased risk of substance-related disorders in children. Children of fathers with the highest alcohol consumption increase were at a 63% higher risk (HR 1.63, 95% CI 1.26–2.12) of substance-related disorders	188
2022	Long-term offspring outcomes	Human (*n*=796)	Observational study	Self-reported alcohol consumption	Preconception paternal alcohol consumption was associated with an increased risk of anxiety and depression in male offspring following at age 4 (RR 1.33, 95% CI 1.09–1.61) and 6 (RR 1.37, 95% CI 1.02–1.85) years	184

aOR, adjusted odds ratio; HR, hazard ratio; OR, odds ratio; RR, risk ratio; SMD, standardized mean difference.

use in the past year, and prevalence was highest (36.6%, ~6.1 million) among those 18–25 years old[47]. With regard to biological plausibility for cannabis negatively influencing male reproductive health, the main psychoactive component of cannabis, Δ^9-tetrahydrocannabinol (THC), binds to receptors in the endocannabinoid system (ECS) that are present in sperm and throughout the male reproductive tract[48] (Box 3). The ECS has various roles in regulating the HPG axis, including control of gonadotropin secretion[49,50], synthesis of testosterone in Leydig cells[51], spermatogenesis[52] and function of sperm[53].

Overall, results of studies on cannabis and male fertility are variable but suggest that cannabis use adversely affects male reproductive health (Table 2). The data support a link between cannabis use and altered male reproductive hormones, semen parameters, libido, and erectile, ejaculation and orgasmic dysfunction[6,7]. The heterogeneity in results, especially in human studies, is partly a result of reliance on retrospective data, diversity of study populations, frequency and potency of cannabis use, confounding variables including polysubstance use, sociodemographic factors, and differences between cannabis products and modes of delivery. Cannabinoids have been shown to have varying effects on different ECS targets; thus, various types of cannabis products might have a diverse range of downstream effects[54]. Limitations of preclinical studies include a focus on acute exposure to cannabis, doses and administration methods that are not representative of human usage, as well as intrinsic differences between rodent and human physiology. This article focuses on the effects of cannabis only; cannabidiol is not discussed as it has a different side effects profile and affinity for cannabinoid receptors[55].

Male reproductive hormones. Mixed results have been reported in preclinical and human studies regarding the effect of cannabis use on the HPG axis and male reproductive hormones. Findings from both preclinical and human studies show decreased, increased, or no effect on testosterone levels[7]. Results of a systematic review of 91 studies (30 clinical studies and 61 animal or in vitro studies) showed that the effect of cannabis use on serum testosterone levels is variable[56]. In 2,074 men undergoing evaluation for infertility, men with primary infertility who reported previous cannabis use had significantly reduced testosterone levels (median (interquartile range) 4.2 ng/ml (3.3–5.6 ng/ml)) compared with non-users (4.6 ng/ml (3.5–5.8 ng/ml)) ($P = 0.03$)[57]. A significant decrease in plasma testosterone levels was also observed in men who chronically used cannabis at least 4 days a week for a minimum of 6 months (416 ± 34 ng per 100 ml) versus those who had never used it (742 ± 29 ng per 100 ml) ($P < 0.001$)[58]. This observation is similar to those in a rhesus macaque model of chronic THC edible consumption (2.5 mg/7 kg/day – equivalent to a heavy medical cannabis dose) that also showed partial reversal of testosterone levels with cessation of THC[59]. By contrast, results of a large population study of 1,215 healthy men aged 18–26 years showed 7% higher serum testosterone levels in men who smoked cannabis regularly more than once per week than men who had never used cannabis[60]. Similarly, in a study in which 2011–2016 National Health and Nutrition Examination Survey data from men in the USA were used, THC use was associated with a small increase in serum testosterone[61]. However, this observed rise in testosterone was not associated with the frequency of THC use, suggesting that a dose-dependent response did not occur[61]. Interestingly, no difference in serum testosterone between men who had previously used cannabis and those who had never used it was shown in other studies[62,63], including a previous study using 2011–2012 National Health and Nutrition Examination Survey data[63]. These findings suggest that cessation of cannabis use can reverse its effects on serum testosterone. Differing reported results could be a result of serum testosterone levels having diurnal fluctuation, peaking in the morning and decreasing throughout the day[64]. Thus, the time of day when blood collection was performed can affect the results.

Box 3

The endocannabinoid system

The endocannabinoid system comprises the main endocannabinoid receptors CB1R (primarily in the central nervous system) and CB2R (primarily in the peripheral nervous system, especially immune cells), which are present throughout the male reproductive tract and in sperm.

The reported effect of cannabis use on FSH levels has also been conflicting. Men who smoked >10 cannabis cigarettes a week had significantly lower FSH levels (7 ± 1 mIU per ml) than those only smoking 5–9 cannabis cigarettes weekly (12 ± 1 mIU per ml) ($P < 0.01$)[58]. Results of a systematic review showed a statistically significant decrease in FSH in men who used cannabis, but the standardized mean difference of 0.142 (95% CI −0.243 to 0.0425, $P = 0.005$) was not felt to be clinically significant[65]. However, because of the limited and heterogenous studies included in the systematic review, an effect of cannabis on testicular function cannot be excluded. By contrast, chronic daily exposure to THC edibles in six male rhesus macaques resulted in a significant increase in FSH ($P = 0.01$) during THC consumption (0.17 ± 0.06 ng/ml to 0.33 ± 0.18 ng/ml) that almost returned to baseline (0.20 ± 0.10 ng/ml) after 4 months of THC cessation[59]. These findings support the benefits of abstaining from THC use for male fertility.

Significantly reduced LH levels ($P < 0.05$) in four men following acute THC exposure from smoking cannabis have been reported[66]. However, a dose-dependent effect of THC on serum LH levels is not apparent, possibly secondary to the small sample size studied. Furthermore, no significant difference in LH levels was observed in 20 men smoking 5–9 versus ≥10 cannabis cigarettes weekly, suggesting that no frequency-dependent effect was noted[58]. Interestingly, significantly increased LH levels following chronic THC edible consumption (0.69 ± 0.18 ng/ml to 1.10 ± 0.35 ng/ml, $P < 0.001$) were observed in a rhesus macaque model[67] that did not return to baseline after discontinuation of THC (0.98 ± 0.60 ng/ml). These findings suggest that cessation of THC might not reverse the effect on LH levels[59].

Overall, cannabis use seems to have variable effects on testosterone, FSH and LH levels and most consistently demonstrates an influence on testosterone levels. This observation highlights the potential association between cannabis use and male fertility. The variability observed is partly caused by the substantial increase in the potency and available formulations of cannabis over the past two decades; thus, comparing studies from different time periods is challenging. The National Institute on Drug Abuse, starting 10 May 2021, has also established 5 mg as the standard research unit for cannabis studies because this dose can produce a ‘high’ in both experienced and occasional users, and in some states it is used as the standard serving size in edible products that contain THC. However, it will be important to also develop a way of comparing different modes of

Table 2 | Effect of cannabis on reproductive health and offspring outcomes

Year	Primary measures	Species	Study design	Conditions	Results	Ref.
Reproductive health						
2019	Male reproductive hormones	Non-human primates and human (7 clinical studies and 23 animal in vitro studies)	Systematic review	In vitro models, mammalian animal studies, and human studies reviewed	Long-term cannabis consumption was not consistently associated with hypothalamic–pituitary–gonadal axis hormones in human clinical studies. Negative or potentially negative effects of cannabis on hormones were noted in non-human and in vitro studies	56
2022	Male reproductive hormones	Human (n=2,074)	Cross-sectional study	Self-reported cannabis use	Men with previous cannabis use had significantly reduced testosterone compared with non-users (P=0.03). No significant relationship with cannabis use and FSH or luteinizing hormone levels was observed	57
1974	Male reproductive hormones and semen parameters	Human (n=20)	Observational study	Self-reported cannabis use	Chronic users had significantly reduced testosterone compared with non-users (P<0.001) and were at an increased risk of oligospermia. Reduced FSH levels were found in men with increased cannabis consumption (P<0.01)	58
2023	Male reproductive hormones, semen parameters, and testicular volume	Non-human primate (n=6 total)	Experimental study	Edible Δ^8-THC 2.5 mg/7 kg/day	Chronic THC consumption was associated with decreased testosterone levels and testicular volume, with partial reversal upon THC cessation. THC use was also associated with increased FSH (P=0.01) that approached baseline upon THC cessation. Last, THC use also decreased semen volume	59
2015	Hormone levels; semen parameters	Human (n=1,215)	Cross-sectional study	Self-reported cannabis use	7% increased testosterone levels in those who smoked cannabis regularly. Cannabis smoking ≥1/week was associated with reduced sperm concentration (P=0.12) and sperm count (P=0.17)	60
2017	Male reproductive hormones	Human (n=1,577)	Cross-sectional study	Self-reported cannabis use	No difference in testosterone levels based on ever using cannabis. Increased testosterone was associated with more recent use, especially in the past month and year	63
2021	Male reproductive hormones and semen parameters	Human (n=4,787)	Systematic revies	Self-reported cannabis use	Significantly decreased FSH in men using cannabis (SMD −0.142), but no significant differences in testosterone or luteinizing hormone. Among cannabis users, 45% had impaired semen parameters versus 25% in non-users; this result did not reach clinical significance (RR 1.16, 95% CI 0.84–1.60; P=0.37)	65
2022	Testicular volume	Non-human primate (n=6)	Experimental study	Edible THC exposure (2.5 mg/7 kg/day)	Chronic daily THC use for 7 months was associated with decreased total bilateral testicular volume with exposure (mean decrease of 58%)	67
2014	Semen parameters	Human (n=1,970)	Observational study	Self-reported cannabis use	Cannabis use in the past 3 months was associated with poor sperm morphology (OR 1.94, 95% CI 1.05–3.60)	70
2020	Semen parameters	Human (n=229)	Cross-sectional study	Self-reported cannabis use	Recent (<6 weeks) users (OR 2.6, 95% CI 1.0–6.8) and heavy (>2 g) users (OR 4.3, 95% CI 1.1–15.9) were at an increased risk of having asthenozoospermia. Moderate users (1–2 g) were at an increased risk of teratozoospermia (OR 3.4, 95% CI 1.5–7.9)	71
2021	Semen parameters	Human (n=409)	Cross-sectional study	Self-reported cannabis use	Current users had increased odds of abnormal sperm morphology (OR 2.15, 95% CI 1.21–3.79), low semen volume (OR 2.76, 95% CI 1.19–6.42) but low odds of reduced motility (OR 0.47, 95% CI 0.25–0.91)	72
1978	Semen parameters	Rat (n=25 total, 9 controls, 4 placebo control, 6 at 0.4 mg/kg THC, and 6 at 3 mg/kg THC)	Experimental study	Cannabis smoke (0.4 mg/kg and 3 mg/kg THC)	Decreased epididymal sperm noted with heavy exposure (3 mg/kg THC), as well as increased dissociation of sperm head and tail	75

Table 2 (continued) | Effect of cannabis on reproductive health and offspring outcomes

Year	Primary measures	Species	Study design	Conditions	Results	Ref.
Reproductive health (continued)						
1978	Semen parameters	Mice (*n* = 69 total, 15 control, 30 at 5 mg/kg, and 24 at 10 mg/kg)	Experimental study	Intraperitoneal injections of cannabinoids (5 mg/kg and 10 mg/kg THC)	Compared with controls, THC-exposed mice had an increased incidence of abnormal sperm and rate of translocations on cytogenetic assessment	76
1979	Semen parameters	Human (*n* = 16)	Observational study	Self-reported cannabis use	Significant decline in sperm concentration and count, as well as reduced motility and normal morphology with high exposure (8–20 cannabis cigarettes/day to cannabis use ($P < 0.01$)	77
1985	Semen parameters	Mice (*n* = 48 males all THC-exposed)	Experimental study	THC (50 mg/kg) given by oral gavage	No significant increase in dominant-lethal or heritable translocation mutations	78
2018	Semen parameters	Human (*n* = 24 total, 12 cannabis users and 12 non-users) Rat	Observational study (human) Experimental study (rat)	Self-reported cannabis use (human) Oral gavage of 2 mg/kg THC for 12 days (rat)	Significant differences in DNA methylation noted between users and non-users in both human and rodent sperm, particularly in Hippo signalling and cancer signalling pathways. Cannabis use compared with non-use was associated with significantly lower sperm concentration (58.1 ± 26.5 vs 96.3 ± 49.7, $P < 0.05$)	80
2006	Semen parameters	Human (*n* = 78)	Experimental study	Sperm incubated with THC at various concentrations (0.032, 0.32 or 4.8 μM)	Dose-dependent decreased motility and spontaneous and induced acrosome reactions noted	80
1977	Semen parameters	Canine (*n* = 10)	Experimental study	Daily administration of cannabis extract (12.5 mg/kg) for 30 days	Complete arrest of spermatogenesis and histological degeneration noted. Testicular weight was significantly lower in those exposed to cannabis than in controls ($P < 0.01$)	81
2011	Semen parameters and testicular volume	Mouse (*n* = 18 total, 6 controls, 6 at 3 mg/kg/day, and 6 at 6 mg/kg/day)	Experimental study	Subjects given cannabis (3 mg/kg/day or 6 mg/kg/day) orally for 36 days	Daily cannabis intake is associated with decreased sperm count, viability and motility, as well as histological degenerative changes of the testes	89
1974	Testicular volume	Mouse (*n* = 40 total, 10 control, 10 at 50 mg, 10 at 90 mg and 10 allowed to recover for 63 days)	Experimental study	Daily intraperitoneal injections of cannabis extract (10 mg/ml, 50 mg in 25 days and 90 mg in 45 days) given for 45 days	Significantly decreased testicular weight (515 mg ± 10 and 534 mg ± 51 with THC exposure versus 697 mg ± 28 control, $P < 0.05$) with shrinkage of the seminiferous tubules (155 μm ± 6 and 148 μm ± 3 with THC exposure vs 196 μm ± 2 control, $P < 0.001$) and seminal vesicles (293 mg ± 20 and 274 mg ± 29 with THC exposure vs 520 mg ± 38 control, $P < 0.001$)	90
1982	Testicular volume	Rat (5 total groups with 8–10 males per group; group 1 at 25 mg/kg and vehicle, group 2 vehicle, group 3 at 25 mg/kg with sesame oil, group 4 sesame oil, and group 5 untreated)	Experimental study	Oral administration of THC (1, 5 and 25 mg/kg/day) and cannabis extracts (3, 15 and 75 mg/kg/day)	Prostate, seminal vesicles and epididymal weights noted to be decreased in those with high dose levels	91
1977	Testicular volume	Rat (*n* = 20 total, 5 THC-treated, 5 cannabidiol treated, 5 vehicle treated control, and 5 untreated control)	Experimental study	Subjects injected intraperitoneally with cannabinoids (2 mg/kg)	77% reduction in testicular weight in those exposed to cannabinoids was noted	92
2018	Hormone levels; semen parameters; testicular volume	Mouse (*n* = 30 total, 10 control, 10 at 15 mg/kg/day and 10 at 30 mg/kg/day)	Experimental study	Cannabinoid (15 and 30 mg/kg/day) administered orally to subjects for 34 days	76% decrease in total circulating testosterone was observed in the high cannabinoid-exposure group, with a decreased number of sperm in the epididymis and increased abnormal morphology. No significant differences in testicular weight were noted	93

Table 2 (continued) | Effect of cannabis on reproductive health and offspring outcomes

Year	Primary measures	Species	Study design	Conditions	Results	Ref.
Reproductive health (continued)						
2010	Testicular volume	Mouse (*n*=60 total, 12 controls, 12 at 50 mg, 12 at 60 mg, 12 at 80 mg, 12 at 80 mg followed by cessation for 45 days)	Experimental study	Intraperitoneal injection of cannabis extract (40–60 mg)	Significant shrinkage of tubular diameter and regression of seminiferous epithelium in testes at low doses was noted (178.67 µm ± 0.98 at a 40-mg dose versus 136.20 µm ± 1.34 at a 60-mg dose versus 230.54 µm ± 3.47 in controls). Withdrawal was associated with an increase in testosterone levels	94
2022	Testicular volume	Human (*n*=316)	Cross-sectional study	Self-reported cannabis use	Cannabis use was not significantly associated with testis volume	98
2008	Sexual function	Human (*n*=71 total, 64 men with erectile dysfunction and 7 healthy men)	Experimental study	Self-reported cannabis use	Chronic cannabis use (at least weekly) can induce early endothelial damage in men with erectile dysfunction compared with non-users (12 ± 6 ml $\min^{-1}$ versus 34 ±5 l $\min^{-1}$, $P=0.003$)	102
2019	Sexual function	Human (*n*=3,395)	Systematic review	Self-reported cannabis use	The odds of erectile dysfunction in cannabis users were significantly increased compared with non-users (OR 3.83, 95% CI 1.30–11.28; $P=0.02$)	103
2004	Sexual function	Human (*n*=3,004)	Cross-sectional study	Self-reported cannabis use disorders	Cannabis use was associated with inhibited orgasm and painful sex	104
2010	Sexual function	Human (*n*=8,650)	Cross-sectional study	Self-reported cannabis use	Daily cannabis use compared with no use was associated with increased odds for ≥2 sexual partners in the prior year. In men, daily cannabis use compared with no use was associated with increased inability to reach orgasm (OR 3.94, 95% CI 1.71–9.07; $P<0.01$), premature orgasm (OR 2.68, 95% CI 1.41–5.08; $P<0.01$) and reaching orgasm too slowly (OR 2.05, 95% CI 1.02–4.12; $P=0.04$)	105
2019	Sexual function	Human (*n*=216)	Cross-sectional study	Online questionnaire querying aspects of sexual experience	52.3% of respondents reported using cannabis to alter sexual experience, with 39% reporting improvement, 16% reporting mixed results and 5% reporting a worse experience. Many participants reported improved relaxation, increased touch sensitivity and intensity of feelings	107
2023	Sexual function	Human (*n*=811 total, 276 men)	Observational study	Self-reported cannabis use	Cannabis use was perceived to increase sexual function and satisfaction, particularly increased desire and orgasm intensity. When using cannabis, 93.4% reported an increased ability to achieve an erection, 92.4% noted an increase in maintaining an erection and 70% reported a slightly or significantly increased orgasm intensity	108
2020	Sexual function	Human (*n*=325)	Cross-sectional study	Self-reported cannabis use	Association between increased frequency of cannabis use and increased male sexual function was noted	109
2017	Sexual function	Human (*n*=22,943)	Cross-sectional study	Self-reported cannabis use	Cannabis users had significantly higher sexual frequency than never users	110
2021	Sexual function	Human (*n*=7,809)	Cross-sectional study	Self-reported cannabis use	Cannabis use was associated with positive ADAM scores (52% versus 46%, $P<0.001$) and increased odds of a positive ADAM score (OR 1.29, 95% CI 1.12–1.48). Cannabis users reported higher sexual frequency (8.8±5.1 events/month versus 7.8±4.9, $P<0.05$)	111
1994	Sexual function	Rat	Experimental study	Single oral dose of THC	Exposure to THC decreased the number of subjects exhibiting copulatory behaviour and increased periods to mounting and intromission	112
2003	Sexual function	Rat	Experimental study	Oral THC over a 30-day period	Subjects exhibited decreased mounting behaviour, as well as decreased sperm count and rates of impregnation	113
2018	Sexual function	Human (*n*=22,943 men)	Observational study	Self-reported cannabis use	Frequent weekly and daily cannabis use among male partners was positively associated with intercourse frequency (IRR 1.22, 95% CI 1.06–1.04, $P=0.006$ and IRR 1.36, 95% CI 1.21–1.53, $P<0.001$, respectively)	114

Table 2 (continued) | Effect of cannabis on reproductive health and offspring outcomes

Year	Primary measures	Species	Study design	Conditions	Results	Ref.
Reproductive health (continued)						
2018	Sexual function	Human ($n=758$)	Cross-sectional study	Self-reported cannabis use	16.5% of men reported using cannabis while attempting to conceive. The ratio of time to pregnancy for non-users versus daily users in men was 1.08 (95% CI 0.79–1.47), showing no significant effect of cannabis use	115
1992	Sexual function	Mouse	Experimental study	10 mg/kg of THC over a 5-week period	1/10 male mice in the THC group failed to mate with the females introduced. One mouse failed to sire offspring	116
1982	Sexual function	Mouse	Experimental study	Oral administration of cannabinoids	Males exposed to cannabidiol impregnated fewer females than those not (60% versus 80% in control animals)	193
2018	Sexual function	Mouse	Experimental study	Oral administration of cannabinoids was performed	Males exposed to cannabinoids exhibited delayed time to first mount and intromission, reduced number of mounts and ejaculation. In the high-dose group, a 30% reduction in fertility was observed and a 23% reduction in the number of litters was observed	242
1974	Sexual function	Human ($n=345$)	Cross-sectional study	Self-reported cannabis use	Variable effects of cannabis on sexual activity were observed, 39.1% of males noted an increase in desire and 59.8% reported an increase in sexual enjoyment. Desire and enjoyment was most increased after smoking one joint compared with smoking two or more joints	242
Offspring outcomes						
1992	Short-term offspring outcomes	Mouse ($n=80$ total, 10 mice per group)	Experimental study	10 mg/kg of THC over a 5-week period	THC administration was not associated with pre-implantation loss or fetal mortality	116
1982	Short-term offspring outcomes	Mouse ($n=72$ total, 18 oil, 18 THC, 18 cannabidiol and 18 cannabinol)	Experimental study	Oral administration of cannabinoids (oil, cannabis extract 25 mg/kg, THC 50 mg/kg and cannabinol 50 mg/kg)	THC exposure in males resulted in significantly more prenatal deaths than in the control group (37% versus 19%, $P<0.05$). Similarly, cannabidiol exposure also resulted in significantly more prenatal and postnatal deaths than in controls (44% and 26% versus 19% and 5%, $P<0.05$). THC and cannabidiol exposure resulted in increased rates of fetal loss	193
2006	Short-term offspring outcomes	Human ($n=221$)	Observational study	Self-reported cannabis use	Lifetime paternal cannabis use of 11–90 times was associated with a 15% decrease in infant birthweight (−0.16, 95% CI −0.31 to −0.01, $P=0.03$) and >90 uses of cannabis was associated with a 23% decrease in infant birthweight (−0.27, 95% CI −0.46 to −0.07, $P=0.01$). Paternal cannabis smoking in the past 15 years was associated with a 16% decrease in infant birthweight (−0.17, −0.33 to −0.02, $P=0.03$)	194
2009	Short-term offspring outcomes	Human ($n=4{,}475$)	Observational study	Self-reported cannabis use	Paternal use of cannabis during pregnancy was not associated with fetal growth rates	195
2021	Short-term offspring outcomes	Human ($n=1{,}535$)	Observational study	Self-reported cannabis use	Paternal cannabis use ≥1 time/week was linked to an increased risk of spontaneous abortion (HR 2.0, 95% CI 1.2–3.1) compared with no use	196
2001	Short-term offspring outcomes	Human ($n=578$)	Case–control study	Self-reported cannabis use	Paternal cannabis use during conception and postnatally was associated with sudden infant death syndrome (OR 2.2, 95% CI 1.2–4.2; $P=0.01$ and OR 2.8, 95% CI 1.1–7.3; $P=0.04$, respectively)	197
2019	Short-term and long-term offspring outcomes	Rats ($n=17$ total, 9 controls and 8 THC-exposed)	Experimental study	Sires were exposed to 2 mg/kg/day of THC for 12 days	THC exposure was associated with long-term impairment in attentional performance in offspring. No significant differences in litter size, birthweight, survival or growth were found	198
2020	Short-term and long-term offspring outcomes	Rats ($n=32$ total, 12 control, 10 at 2 mg/kg/day and 10 at 4 mg/kg/day	Experimental study	THC (0, 2 or 4 mg/kg/day) administered subcutaneously for 28 days	Offspring of male rats exposed to THC showed significant locomotor hyperactivity ($F(2,29)=3.37$, $P<0.05$). These offspring also showed decreased rates of interest in novel object recognition and radial-arm maze tasks	199

Table 2 (continued) | Effect of cannabis on reproductive health and offspring outcomes

Year	Primary measures	Species	Study design	Conditions	Results	Ref.
Offspring outcomes (continued)						
2019	Short-term and long-term offspring outcomes	Human (n=5,903)	Observational study	Self-reported cannabis use	Paternal cannabis use was associated with behavioural issues when using teacher-reported evaluations of children	200
2022	Short-term and long-term offspring outcomes	Rat (n=36 total, three groups with n=12 per group)	Experimental study	THC 4 mg/kg/day injected intraperitoneally	Significant methylation changes in F0 sperm were also seen in F1 sperm, including in the genes *Pxylp1* and *Mtss1l*. Offspring of rats exposed to cannabis had significantly increased rates of cardiomegaly (one-factor ANOVA (P=0.0039)). Post hoc tests showed significantly increased heart weight relative to controls for both the early exposure offspring (P=0.0013) and the late exposure offspring (P=0.0099)	202
1998	Short-term and long-term offspring outcomes	Human (n=7,868)	Case–control study	Self-reported cannabis use	The attributable fraction for paternal cannabis usage in the transposition of the great arteries with an intact ventricular septum was found to be 7.8%	203
1997	Short-term and long-term offspring outcomes	Human (n=4,190)	Case–control study	Self-reported cannabis use	Paternal cannabis usage was significantly associated with ventricular septal defects (OR 1.36, 95% CI 1.05–1.76)	204
2002	Congenital anomalies	Human (n=3,627)	Case–control study	Self-reported cannabis use	Paternal cannabis use was associated with a single ventricle	205
1993	Congenital anomalies	Human (n=644)	Case–control study	Self-reported cannabis use	Paternal preconception cannabis use was associated with an increased risk of rhabdomyosarcoma (RR 2.0, 95% CI 1.3–3.3)	207
2020	Short-term and long-term offspring outcomes	Human (n=24) Rat (n=15)	Case–control, experimental	Self-reported cannabis use (human) Subcutaneously injected 4 mg/kg THC (rat)	Cannabis use is associated with substantial DNA methylation alterations, including in *DLGAP2*, a gene strongly implicated in autism. Offspring of male rats exposed to THC still showed differences in DNA methylation for *Dlgap2* within the nucleus accumbens	208
2018	Short-term and long-term offspring outcomes	Human (n=136)	Observational study	Self-reported cannabis use	DNA methylation status in seven sites of *ANNK1*, *CNR1*, *DRD2* and *NCAM1* genes was examined. Increased rates of methylation in cannabis users were observed in two of the regions assessed: exon 8 of *DRD2* (P=0.034) and the CpG-rich region of *NCAM1* (P=0.0004), both genes involved in the dopaminergic pathway that can be associated with substance use. Other regions assessed showed no statistically significant differences between cannabis users and non-users	210

ADAM, androgen deficiency in the ageing male; FSH, follicle-stimulating hormone; HR, hazard ratio; IRR, incidence rate ratio; OR, odds ratio; RR, risk ratio; SMD, standardized mean difference; THC, tetrahydrocannabinol.

cannabis delivery given that the drug is variable depending on the mode of delivery.

Semen parameters. Human sperm have been shown to express major receptors involved in the ECS, namely cannabinoid type 1 (ref. 68) and type 2 receptors[69] (CB1R, CB2R) and transient receptor potential vanilloid 1 (TRPV1)[53]. Results of in vitro studies have shown the decreased motility and fertilizing ability of human sperm via CB1R activation[68] and decreased motility via CB2R activation[69]. Associations have been observed between cannabis use and abnormal sperm morphology[65,70–72], decreased volume[72], decreased sperm count and concentration[7,58,60,73], and decreased motility[71]. However, these findings were not consistently reported across studies and a systematic review[65] did not demonstrate a significant relationship between cannabis use and abnormal semen parameters. The conflicting results are probably in part because of the high variability in semen analysis results within the same human subject[74].

Results of existing animal[75,76] and human studies[70,77] have suggested that cannabis use alters sperm morphology, but the literature is variable. Examination of lifestyle factors associated with poor sperm morphology in men recruited from fertility clinics across the UK[70] showed that men aged ≤30 years who used cannabis 3 months before sample collection had an increased likelihood of having abnormal (<4% normal) sperm morphology (OR 1.94, 95% CI 1.05–3.60)[70]. By contrast, no significant change in sperm morphology from rhesus macaques was observed following daily THC (2.5 mg/7 kg/day) edible consumption for 7 months, but a dose-dependent increase in sperm DNA fragmentation, a marker of sperm DNA integrity important for fertilization and for the development of healthy offspring, that partially reversed following 4 months of THC cessation, was observed[59]. In a previous mouse study in which mice were exposed to high doses of THC (50 mg/kg) five times weekly for 6 weeks, no increase in lethal mutations or heritable translocations in offspring were found[78]. This observation suggests that THC is not an efficient inducer of chromosome breakage in germ cells of male mice.

A strong association has been observed between cannabis exposure and decreased sperm count and concentration. An association between cannabis use and decreased sperm counts[58,60,79], and reduced sperm counts in individuals who used THC weekly compared with those who have never used THC, has been observed in several human studies[60,80]. In 16 healthy, chronic cannabis smokers with daily or near daily use over months to years, 4 weeks of high-dose cannabis use (8–20 cigarettes/day) resulted in a significant decrease in sperm concentration during the fifth (65% of baseline, $P < 0.001$) and sixth (70% of baseline, $P < 0.01$) weeks after exposure[79]. The underlying aetiology is not well defined, but the observed cannabis-induced reduction in sperm count and concentration has been linked to arrested spermatogenesis[73]. In a previous study, high doses of cannabis (12.5 mg of THC per kilogram of body weight) in canines over 30 days resulted in the total arrest of spermatogenesis[81]. This observation suggests that cannabis at high doses daily for only a month can induce infertility.

In addition to altering sperm count and morphology, cannabis use has been reported to influence sperm motility. Following 4 weeks of exposure to high-dose cannabis (8–20 cigarettes with 2% THC per day), 16 healthy men, who smoked cannabis chronically, had a reduction in sperm motility that improved following cessation of chronic smoking, suggesting the potential for reversibility[79]. Chronic cannabis use is typically defined as daily use for months to years[82]. This effect was similarly observed using collected sperm incubated with THC at concentrations equivalent to therapeutic (0.032 μM) and recreational (0.32 μM and 4.8 μM) plasma levels[83], and was thought to be secondary to decreased mitochondrial transmembrane potential, mediated by CB1R activation[84]. Results of previous studies have demonstrated CB1R on human sperm, showing that THC is a strong exogenous agonist for the CB1R, and observed that a reduction in mitochondrial function, or altered membrane potential, is associated with decreased sperm motility[83,85,86].

Overall, the evidence suggests that cannabis exposure can influence semen parameters, especially sperm count and concentration. However, the reported findings are inconsistent, potentially because in most studies only a single ejaculate was analysed, which might not reflect the degree of variation observed between multiple semen analyses in a single participant[40,67]. Owing to variation between semen collections, a second semen analysis is recommended by the WHO Laboratory Manual for the examination and processing of human semen to improve diagnostic reliability in the evaluation of male fertility[87,88]. Moreover, THC exposure has been variable in many studies, which will confound the true effect of THC on semen parameters. Thus, caution should be taken when comparing studies using single versus multiple ejaculates to assess semen parameters, and future research should evaluate semen characteristics over multiple collections.

Testicular volume. Preclinical studies in dogs and rhesus macaques have demonstrated an association between cannabis use and decreased testicular volume[67,81], in addition to reduced prostate and seminal vesical weight[7,89–91]. Variable effects of cannabis exposure on testicular volume in mice and rats have also been reported[92,93], including histological changes such as degradation of seminiferous tubules[89], which can result in impaired sperm production, with only partial recovery after cannabis cessation[7,94]. The underlying mechanism for these changes in testicular volume is unknown, but has been previously linked to oxidative stress[94,95]. Testicular tissue and the male reproductive system are sensitive to oxidative stress, a major factor in the aetiology of male infertility[96,97].

In rhesus macaques, chronic daily exposure to THC significantly lowered testicular volume by 11.8 cm^3 (95% CI 8.3–15.4, $P < 0.001$) for every mg/7 kg/day increase in THC dosing[67]. This observation was partly caused by decreased seminiferous tubule diameter and germ cell layers on histology[67] that was partially reversed with cessation of THC[59]. As the seminiferous tubules and germ cells are the site of spermatogenesis, this reduced diameter can affect sperm production, highlighting the potential benefit of stopping THC use to reverse these effects. In men aged 18–60 years, associations between regular cannabis use and decreased testicular volume have not been conclusively shown[98]. However, the study was limited by a cross-sectional study design and lacked a matched control group of fertile 'healthy' men[98].

Overall, animal models have consistently demonstrated that cannabis use is linked to testicular atrophy, in part owing to decreased seminiferous tubule diameter and germ cell layers. These effects seem to be partly reversible with abstinence from THC, but studies with increased intervals of THC cessation are needed to determine whether full recovery is possible. Thus, health-care providers should discuss the potential benefit of cessation for at least several months before conception in order to optimize fertility with their patients who are using cannabis and interested in conceiving.

Erectile dysfunction and sexual function. The ECS has been shown to have a role in erectile signalling and capacity[73] and erectile dysfunction[7,58,99]. Results of a study in rats demonstrated that an erection can be induced with administration of rimonabant, a cannabinoid receptor antagonist[100], secondary to activation of neuronal nitric oxide synthase in paraventricular oxytocinergic neurons mediating penile erection[101]. Results of other studies using veno-occlusive plesmography to evaluate endothelium-dependent dilation of arteries in men with erectile dysfunction have suggested that THC might induce erectile dysfunction secondary to early endothelial damage[102]. Veno-occlusive studies revealed impaired endothelium-dependent vasodilatation in men with erectile dysfunction who used cannabis regularly (at least weekly) compared with non-users (12 ± 6 ml min^{-1} versus 34 ± 5 ml min^{-1}, $P = 0.003$)[102]. These findings indicate that men who use cannabis weekly or more frequently have impaired epithelium-dependent vasodilation compared with non-users, which can be modifiable with lifestyle changes, including discontinuing or reducing cannabis use[102]. Results of a systematic review and meta-analysis examining in which the prevalence and risk of erectile dysfunction in cannabis users versus non-users, including five case–control studies, showed that the odds ratio of erectile dysfunction in cannabis users was nearly four times that of non-users (OR 3.83, 95% CI 1.30–11.28, $P = 0.02$)[103]. The study had high heterogeneity ($I^2 = 90\%$), but the results suggest that men with erectile dysfunction would benefit from discontinuing cannabis use. Similarly, results of other large survey studies in which the association between cannabis use and different sexual health outcomes was examined have also found associations between cannabis use and orgasmic dysfunction[104,105]. In one study, a telephone survey of 4,350 Australian men aged 16–64 years was conducted and showed that daily cannabis use versus no use was associated with increased inability to reach orgasm (OR 3.94, 95% CI 1.71–9.07, $P < 0.01$), reaching orgasm too quickly (OR 2.68, 95% CI 1.02–4.12, $P = 0.04$), and too slowly (OR 2.05, 95% CI 1.02–4.12, $P = 0.04$)[105]. The results of this study support that frequent cannabis use can influence the ability to orgasm as desired.

The effects of cannabis on human sexual function are complex and not conclusive, but some evidence suggests a role of the ECS in sexual function. Cannabis extracts have been commonly used to enhance sexual experience[106,107]. In a study including 276 male cannabis users aged 18–85 years, 93.4% reported no change or an increased ability to achieve an erection with cannabis use, 92.4% noted no change or an increase in maintaining an erection when using cannabis, and 70% found that cannabis use slightly or significantly increased orgasm intensity[108]. Results of a survey of 325 men visiting a cannabis dispensary showed that users had higher erectile function scores and satisfaction with intercourse than non-users[109]. Those who used at least 3–5 times a week had a significantly higher International Index of Erectile Function (IIEF) score and intercourse satisfaction than those who used no more than twice a week (65.3 ± 68.02 versus 60.52 ± 13.84, $P = 0.001$ and 12.42 ± 2.26 versus 11.31 ± 3.37, $P = 0.006$, respectively)[109]. Increased rates of coital frequency have been associated with cannabis use compared with never use in a large cohort of 22,943 men (IRR 1.08, 95% CI 1.05–1.11, $P < 0.001$)[110]. Cannabis use in 993 men compared with 6,816 non-users has been linked to increased sexual frequency (8.8 events/month versus 7.8 events/month, $P < 0.05$), that is, not clinically significant[111]. Conversely, in rats, both acute and chronic cannabis exposure have shown to decrease libido and copulatory behaviour[112,113]. The conflicting results observed in humans compared with rats might be because of the much higher THC exposure in rats (5–10 g/kg)[112,113].

Some evidence is suggestive of a link between frequent cannabis use and difficulty achieving orgasm. In a survey of 8,656 men daily cannabis users were more likely to have orgasmic disorders, such as reaching orgasm too quickly (OR 2.68, 95% CI 1.41–5.08, $P < 0.01$), too slowly (OR 2.05, 95% CI 1.02–4.12, $P = 0.04$) or inability to orgasm (OR 3.94, 95% CI 1.71–9.07, $P < 0.01$), than non-users[105]. Chronic cannabis use can result in increased orgasmic disorders, overall cannabis use has been linked to improved sexual experience secondary to increased erectile function and increased coital frequency.

In large human cohort studies, no relation between male cannabis use and fecundity has been found[114,115]. A prior study including 758 men participating in the National Survey of Family Growth across 121 geographic areas in the USA, time ratio to pregnancy of never users versus daily cannabis users was 1.08 (95% CI 0.79–1.47, $P = 0.65$)[115]. Similarly, in another study including 1,125 men at least 21 years old from the Pregnancy Study Online, a prospective cohort of North American couples, little association was found between male cannabis use and fecundability[114]. The fecundability ratio, a ratio of fecundability in each exposure category compared with the reference category, for male cannabis use <1 and ≥1 time a week versus non-use is 0.87 (95% CI 0.66–1.15) and 1.24 (95% CI 0.90–1.70) respectively[114]. Similarly, in studies in mice, no difference in the number of fetuses sired per pregnancy in controls (12.0 ± 0.64) compared with mice administered 10 mg/kg THC orally every 2 days for 5 weeks (12.7 ± 1.41)[116]. These data indicate that cannabis use does not seem to affect fecundity, but these the effects of regular, heavy cannabis use for months to over a year were not examined.

The existing evidence suggests that cannabis has a dose-dependent effect on erectile dysfunction and sexual function. Use of high doses of cannabis in humans is linked to worsened erectile dysfunction and difficulty achieving orgasm, whereas low doses of cannabis are associated with increased sexual desire, frequency and rates of masturbation. Given the complex effects of cannabis use on erectile dysfunction and sexual function, patients must be appropriately counselled to make evidence-informed lifestyle decisions.

Summary. In general, cannabis use, especially heavy and chronic use, negatively affects male reproductive health, but does not seem to influence fecundity in human cohorts (Table 2). Chronic or regular cannabis use is often defined as daily use for months to years, and heavy use is typically associated with daily or more frequent use[82]. Specifically, cannabis use has been linked to altered male reproductive hormones (such as testosterone, FSH and LH), semen parameters, testicular volume and sexual function (for example, libido and erectile dysfunction). For most individuals, cannabis will probably not affect their ability to conceive, but in those with subfertility or infertility, it could be a contributing factor and also exacerbate existing infertility issues, compounding their difficulty to conceive. The evidence also suggests that the effects seem to be dose dependent and potentially reversible[59,67]. This observation is concerning given that the potency of cannabis products has risen dramatically over the last few decades[117]. Thus, health-care providers should counsel men of reproductive age and those interested in conceiving towards cessation to avoid all potential risks, and to encourage reduced cannabis use in patients who are unable to abstain.

Opioids

Over the previous two decades, the use of opioids has risen dramatically across the USA and Canada, and abuse of fentanyl and synthetic, illicit opioids, has increased[118,119]. North America remains the centre of the opioid epidemic, but it has also become a public health issue for other countries, including the UK and Australia[118,119]. In 2015, 37% of American adults had been prescribed at least one opioid pain reliever, a rate that had tripled since the late 1990s[120]. Overdoses, abuse and mortality has increased among many populations, including men of reproductive age[121]. Accompanying these concerns, increasing evidence suggests that opioid use can affect male reproductive health (Table 3). Endogenous opioid peptides are present in the male reproductive tract and are involved in regulating reproductive physiology[122]. Opioids, whether produced endogenously or exogenously, bind to opioid receptors in the hypothalamus, pituitary and testis and influence their function[123]. Data are limited on whether a dose-dependent effect occurs, especially for the short-term use of opioids. Chronic, long-acting opioid use has been linked with an increased risk of androgen suppression compared with similarly dosed short-acting opioids[124,125]. A previous retrospective case–control analysis including 357 men (94 chronic opioid users with at least 90 days of opioid use and 263 non-users) showed a significant, positive linear association between chronic opioid dose and the odds of developing hypogonadism in males (OR 1.44, 95% CI 1.16–1.78, $P < 0.001$)[125]. This observation suggests an increased risk of opioid-induced androgen deficiency and a negative effect on patient quality of life with chronic opioid use. Thus, an increased index of suspicion can result in earlier recognition of symptoms in patients with chronic opioid use.

Male reproductive hormones. In tandem with the drastic rise in opioid use, the incidence of opioid-associated androgen deficiency has increased[126]. Chronic opioid use results in suppression of the HPG axis through its central and peripheral effects, which results in secondary testosterone deficiency or opioid-associated androgen deficiency[126–128]. Opioids act directly on the hypothalamus by binding G protein coupled μ-opioid receptors and inhibiting the pulsatile release of GnRH[126],

Table 3 | Effect of opioids on reproductive health and offspring outcomes

Year	Primary measure	Species	Study design	Conditions	Results	Ref.
Reproductive health						
2013	Reproductive hormones	Humans (*n*=374)	Observational study	Self-reported opioid use	Opioid use was associated with decreased testosterone levels and sperm parameters, including motility, density and morphology, but no change in FSH and LH. Opioid use was linked to increased sperm DNA fragmentation	130
2018	Semen parameters and reproductive hormones	Humans (*n*=60)	Case–control study	Self-reported opioid use	Tramadol use was associated with elevated FSH, LH and prolactin, decreased androgen levels, low-quality semen profiles, decreased sperm viability and progressive motility, and increased incidences of leukocytospermia and abnormal sperm morphology	131
2016	Semen parameters and reproductive hormones	Mouse (*n*=48, 8 groups with 6 animals in each group)	Experimental study	Morphine was intraperitoneally administered at 10 mg/kg daily on day 1 and then increased 2 mg/kg per day on days 2–30	Morphine administration decreased testosterone, LH, FSH, testis weight and sperm parameters, such as count, viability, morphology and motility	135
2017	Erectile dysfunction and sexual function	Human (*n*=8,829)	Systematic review and meta-analysis	Self-reported opioid use	Opioid use was associated with an increased risk of erectile dysfunction	138
2018	Sexual function	Human (*n*=514)	Cross-sectional study	Self-reported opioid use	Chronic opioid use diminished libido and impaired sexual performance	140
Offspring outcomes						
2021	Long-term offspring outcomes	Human (*n*=8,410)	Population-based study	Self-reported opioid use	Regular preconception paternal opioid use was independently associated with increased odds of overweight or obesity in offspring (aOR 1.76, 95% CI 1.15–2.71)	213
2018	Short-term and long-term offspring outcomes	Rat (*n*=36, 19 morphine exposed and 17 saline exposed)	Experimental study	Escalating doses of morphine (2.5 mg/kg/day increased by 2.5 mg/kg per day to 25 mg/kg/day) for 10 days during postnatal days 31–40. Mating occurred 20 days after the last morphine injection	Transgenerational effect of paternal morphine exposure on adolescent offspring pain perception and the antinociceptive effect of morphine	214
2021	Short-term and long-term offspring outcomes	Rat (for F0, saline-treated *n*=18, morphine treated *n*=16; for F1, *n*=14 and *n*=11 for the saline-sired and morphine-sired group, respectively)	Experimental study	Escalating doses of morphine (2.5–25 mg/kg, subcutaneous injection) for 10 days	Paternal opiate exposure before conception impaired inhibitory control in male progeny; offspring exhibited delayed learning and impulsive behaviour	215
2020	Short-term and long-term offspring outcomes	Rat (for sires, *n*=40 control and *n*=39 morphine; for dams, *n*=55 control and *n*=58 morphine; offspring used in experiments varied between experimental approaches but was *n*=8–14 animals per group)	Experimental study	Self-administered morphine	Paternal morphine exposure selectively disrupted novel object recognition in female but not male progeny, and did not change anxiety-like behaviour or stress-induced hypothalamic–pituitary–adrenal axis activation in both male and female offspring	216
1991	Short-term and long-term offspring outcomes	Rat (*n*=70 total, 10 animals for each group — weeks 1–6 — and 20 animals for week 9	Experimental study	Implants with morphine (75 mg) or placebo pellets initially one on day 1, then two pellets at 4, 7 and 10 days. Pellets remain present for the following intervals: 1, 2, 3, 4, 6 and 9 weeks	Data suggest that paternal exposure to opiates influences offspring sexual maturation and can also have long-term, selective and gender-specific effects on endocrine function in their male offspring	218

Table 3 (continued) | Effect of opioids on reproductive health and offspring outcomes

Year	Primary measure	Species	Study design	Conditions	Results	Ref.
Offspring outcomes (continued)						
2022	Short-term offspring outcomes	Rat (*n*=20 Sprague–Dawley, 10 males and 10 females; and *n*=20 Long–Evans, 10 males and 10 females were used) For multigenerational studies 18 Sprague–Dawley rats (9 males and 9 females) were used to produce a total of 112 F1 pups (males: 28 saline sired, 32 morphine sired; females: 22 saline sired, 30 morphine sired). Animals were randomly assigned to groups, with two to four rats from a single litter used in the studies)	Experimental study	Self-administered morphine 3 mg/kg morphine sulfate in sterile 0.9% saline	Paternal preconception exposure to opioids increased the sensitivity of male offspring to the pain-relieving effects of morphine that correlated with gene expression changes within the regulator of G protein signalling family proteins	221
2017	Short-term and long-term offspring outcomes	Rat (*n*=24 total, 12 morphine exposed and 12 saline exposed)	Experimental study	Morphine (10 mg/kg) or saline injected twice daily for 2 weeks	Paternal morphine exposure was associated with significantly higher anxiety-like behaviour and enhanced voluntary consumption of morphine in morphine-derived compared with saline-derived male offspring ($P<0.01$)	220

aOR, adjusted odds ratio; FSH, follicle-stimulating hormone; LH, luteinizing hormone.

blocking the release of LH and FSH by the pituitary gland, and subsequently affecting testicular testosterone production[126]. This syndrome is characterized by low levels of FSH and LH, leading to inadequate production of sex hormones, namely testosterone. A decrease in sex hormone levels, or interference in the pulsatory secretion of GnRH at the hypothalamic level, and the subsequent reduction in FSH and LH hormone secretion from the pituitary gland, are some known effects of opioids on the reproduction system[129]. In men ($n = 186$, aged 20–50 years) using opioids, a negative effect of opioid use on testosterone levels was observed, but no change in serum FSH and LH levels was observed[130]. Altered hormonal profiles were also noted in men ($n = 30$) abusing tramadol compared with age-matched non-users ($n = 30$), such as elevated FSH, LH and prolactin, and decreased androgen levels[131]. These results suggest that opioid use can affect male reproductive hormones and negatively affect fertility, including time to conception.

Overall, opioid use is most consistently associated with decreased testosterone levels but has a more variable effect on other male reproductive hormones, such as FSH, LH and prolactin. The different types of opioid drugs and interindividual variability in hormone levels have probably contributed to the heterogeneity in the current data[35,36]. To overcome the limitations of the existing research, animal models with strong translational strength are needed to study the dose-dependent effect of chronic opioid use on male reproductive hormones and fertility potential.

Semen parameters. Opioids have been shown to increase free radical production, which can adversely affect spermatogenesis[132]. In a case–control study men aged 20–40 years with morphine dependency ($n = 30$) had a significant decrease in both progressive (35.76% ± 15.95% versus 58.3% ± 10.93%, $P = 0.038$) and total sperm motility (46.13% ± 17.5% versus 71.36% ± 8.38%, $P < 0.0001$) compared with healthy men ($n = 30$))[132]. In addition, morphine dependence was associated with decreased sperm chromatin condensation and increased rates of sperm apoptosis, although statistical significance was not observed[132]. In a study investigating fertility and potential correlations with opioid abuse, opioid use correlated with decreased sperm parameters, including motility and morphology[130]. Increased DNA fragmentation in patients using morphine was also observed. Similarly, in another study, long-term users of tramadol had low-quality semen profiles, including low sperm viability, decreased progressive motility, increased incidence of leukocytospermia, and abnormal sperm morphology[131]. Taken together, these findings suggest that regular opioid exposure adversely affects male fertility and follow-up studies are needed to determine if these effects are reversible with opioid abstinence.

Morphine has been shown to increase blood nitric oxide by regulating intracellular calcium and activating calcium/calmodulin-dependent nitric oxide synthase[133]. A correlation between nitric oxide and sperm acrosome and tail defects in mice and humans has been observed, showing that nitric oxide reduces sperm motility by decreasing adenosine triphosphate (ATP) levels[134]. Nitric oxide can also impair mitochondrial membranes in sperm, stimulating apoptosis[135]. These observations suggest that morphine exposure can adversely affect sperm function and fertility through an inhibitory effect of nitric oxide on cellular respiration[134].

In general, opioid drug exposure, especially morphine dependency, is associated with an adverse effect on semen characteristics, including sperm count, motility, morphology and viability. The mechanism is not clear; however, reduced testosterone levels from opioid use have a role. For individuals interested in conceiving, discussing the potential influence that opioids have on semen parameters is important and abstaining or reducing the quantity of opioids used should be considered.

Testicular volume. Data regarding the effect of opioid exposure on testicular volume are lacking. Results of a case–control study of 100 men with opioid dependence and 100 healthy men showed that opioid use was associated with significantly reduced testicular volumes

(11.2 ± 2.2 and 25.1 ± 2.7 cm^3, $P < 0.001$)[136]. The underlying aetiology for this effect is unknown, but might be partly caused by opioid-induced alterations in male reproductive hormones, including decreased testosterone and altered LH and FSH levels. These alterations can result in decreased testicular growth because LH and FSH both have a crucial role in maintaining spermatogenesis[127,128]. The scant existing literature is probably because individuals with opioid dependence are less likely to be undergoing a fertility work-up, and testicular volume evaluation is not usually a part of the initial clinical assessment. Future research studies should include assessment of testicular volume when examining the effect of opioid use on male fertility and also assess the underlying mechanisms for the observed decrease in testicular size.

Erectile dysfunction and sexual function. Current studies consistently report a high prevalence of erectile dysfunction among males with opioid dependence[137,138], partly caused by the inhibition of the HPG axis and decrease in testosterone levels[127,128] Erectile dysfunction is multifactorial and other potential contributing factors include depression, atherosclerosis, obesity, diabetes and trauma[139]. Results of a systematic review and meta-analysis of 8,829 men from 10 studies showed that opioid use was associated with an increased risk of erectile dysfunction (risk ratio (RR) 1.96, 95% CI 1.66–2.32, $P < 0.001$)[138]. In another study including 63 men aged 30–50 years with an average of 11.9 ± 9.4 years of opioid use, 34% reported erectile dysfunction[137]. Common sequelae of chronic opioid use also include diminished libido and impaired sexual performance; thus, health-care providers need to discuss with their patients when prescribing opioids[140].

Summary. In general, studies examining the effect of opioid use on male reproductive health are limited compared with other types of substance use and more studies are needed to guide evidence-based recommendations. The existing data suggest that opioid use is associated with a negative effect on testosterone, semen parameters, testicular volume and sexual function, including increased erectile dysfunction and decreased libido (Table 3). Based on the current safety data, health-care providers should discuss with patients interested in conceiving the potential risks of opioid use to male fertility, including increased difficulty getting pregnant, recommend discontinuation of opioids before conception, and consider safer alternatives.

Nicotine

In 2019, the global number of current tobacco users was estimated to be one billion[141], making tobacco the second most commonly used psychoactive substance worldwide[142]. Over the past decade, diversified commercial nicotine delivery systems or nicotine products (such as e-liquids, smokeless tobacco pouches and nicotine replacement therapies) have become increasingly popular. However, most of the world's nicotine exposure is still from combustible tobacco smoking[143], making nicotine and tobacco smoke a prevalent public health concern. Exposure to nicotine products has been shown to affect the male reproductive system directly by altering the testis and sperm, and indirectly by altering the endocrine system[144,145] (Table 4).

Male reproductive hormones. The evidence concerning the effects of tobacco smoke on male reproductive hormones is conflicting. In one study, serum levels of FSH, LH and testosterone among 126 non-smoking men and 178 men who smoked cigarettes for over 6 months (98 men smoked 1–20 cigarettes per day, 80 men smoked >21 cigarettes per day) showed that heavy smokers (>20 cigarettes per day) had significantly lower testosterone levels ($P < 0.0001$) and significantly higher FSH ($P < 0.0001$) and LH levels ($P < 0.001$) than non-smokers[144]. These findings suggest that heavy smoking can be associated with impaired male fertility, an elevated FSH is indicative of abnormal spermatogenesis and primary testicular failure. Conversely, in 95 men who smoked cigarettes (30 mild smokers: <5 cigarettes per day, 30 moderate smokers: 5–10 cigarettes per day, 35 heavy smokers: >10 cigarettes per day), smoking significantly reduced both total testosterone and FSH in a dose-dependent fashion compared with 45 non-smoking men[146]. The results of this study demonstrated that a reduction in total testosterone (18.8 nmol/l ± 4.6 versus 14.9 nmol/l ± 3.2 versus 14.5 nmol/l ± 5.7 versus 0.24 nmol/l ± 0.1, $P = 0.0.21$) and FSH (8.56 IU/l ± 2.8 versus 6.90 IU/l ± 2.6 versus 6.80 IU/l ± 2.7 versus 5.80 IU/l ± 2.1, $P = 0.007$) was inversely proportional to the number of cigarettes smoked per day (non-smoker, mild, moderate and heavy). These observed findings suggest that in men who are unable to quit smoking, cutting down on the number of cigarettes smoked might be beneficial to their fertility. By contrast, in another study[147] including 90 men who smoked cigarettes and 165 men who did not (aged 30–70 years), no difference in LH ($P = 0.573$), FSH ($P = 0.693$) or total testosterone ($P = 0.580$) was observed, regardless of pack-years of smoking; whereas, in 3,427 men who smoked cigarettes, total testosterone increased 15% compared with men who did not smoke ($P < 0.001$)[148]. These inconsistent outcomes across studies provide challenges to counselling patients regarding the potential risks of cigarette smoking and male fertility.

To determine whether tobacco cessation is beneficial, cessation of smoking for 1 year was analysed in 76 men who smoked cigarettes (average 24 ± 10 cigarettes per day) and found no significant differences in testosterone, dehydroepiandrosterone, dehydroepiandrosterone sulphate, cortisol or sex hormone-binding globulin during a 1-year cessation from smoking[149]. This observation suggests that impaired male fertility associated with chronic cigarette smoking might not be reversible with cessation.

Overall, the scientific literature focused on nicotine use and male reproductive hormones is heterogenous but suggest a potential effect on testosterone, FSH and LH with heavy smoking that might not be reversible with smoking cessation. This possibility highlights the importance of increased public awareness and health-care provider counselling regarding the potential adverse effect on male fertility associated with smoking and the benefit of smoking abstinence or limiting the amount smoked. The conflicting results are partly caused by the range of tobacco products available and the diurnal variation of male reproductive hormone levels[35]. Relevant animal models with strong translation are needed to address this by limiting confounding variables, controlling the amount of tobacco exposure and timing hormonal assessments. Ideally, this research would be performed using a single-case study design in which each subject can serve as their own control to minimize inter-animal variability.

Semen parameters. Many studies within the last three decades have shown positive correlations between nicotine and cigarette smoke exposure and altered semen parameters[150–152]. Existing evidence also largely demonstrates that exposure to tobacco smoking increases sperm DNA fragmentation, which has been linked to the polycyclic aromatic hydrocarbons present in tobacco smoke[153–155]. In a meta-analysis of 20 studies including 5,865 male participants (at least 13 years of age), exposure to cigarette smoke was significantly associated with reductions in sperm count (MD 9.72×10^6ml, 95% CI 13.32–6.12, $P < 0.001$), sperm motility (MD 3.48%, 95% CI 5.53–1.44, $P < 0.001$) and

Table 4 | Effect of nicotine on reproductive health and offspring outcomes

Year	Primary measure	Species	Study design	Conditions	Results	Ref.
Reproductive health						
2012	Male reproductive hormones	Human (n=304)	Observational study	Self-reported smoking	Cigarette smoking was associated with increased morphological defects ($P<0.0001$), reduced motility ($P<0.001$), decreased sperm DNA integrity ($P=0.006$), endocrine hormonal status, and the number of CAG repeats in the androgen receptor gene	144
2016	Male reproductive hormones	Human (n=140)	Observational study	Self-reported smoking	Mild, moderate and heavy smokers displayed significant decreases in semen volume (2.7±0.7, 2.2±0.04, 2.6±0.3 versus 3.37±1.0) free (0.27±0.1, 0.26±0.1, 0.24±0.1 versus 0.36±0.1) and total testosterone (14.9±3.2, 14.5±5.7, 13.8±5.6 versus 18.8±4.6), follicle-stimulating hormone (6.9±2.6, 6.8±2.7, 5.8±2.1 versus 8.56±2.8) and sperm counts (sperm/ml 73.6±21.6, 64.3±19.8, 24.5±12.1 versus 93.17±22.1) motility (% immobile 35.1±9.8, 36.2±8.7, 34.5±10.1 versus 7.64±2.3) and morphology compared with non-smokers. No significant difference in luteinizing hormone, E_2 and prolactin was observed. Smokers also had an increased risk of developing oligospermia (OR 3.1, $P=0.047$), asthenozoospermia (OR 4.2, $P=0.001$) and teratozoospermia (OR 4.7, $P=0.0003$)	146
2009	Male reproductive hormones	Human (n=255)	Cross-sectional study	Self-reported smoking	Non-smokers and smokers displayed similar mean values for androgens, gonadotropins and sex hormone binding globulin	147
2007	Male reproductive hormones	Human (n=3,427)	Observational study	Self-reported smoking	Significantly increased levels of total and free testosterone were found in smokers. Smokers had 15% higher total ($P<0.001$) and 13% higher free ($P<0.01$) testosterone levels than men who have never smoked	148
2013	Male reproductive hormones	Human (n=76)	Observational study	Self-reported smoking and cotinine levels	Smoking cessation was not associated with a significant change in male total testosterone and sex hormone binding globulin levels	149
2016	Semen parameters	Human (n=5,865)	Systematic review and meta-analysis	Self-reported smoking Cigarettes/day: mild (1–10); moderate (10–20); heavy (>20)	Smoking was associated with reduced sperm count (MD -9.72×10^{6}/ml, 95% CI −13.32 to −6.12), motility (−3.48%, 95% CI −5.53 to −1.44) and morphology (−1.37%, 95% CI −2.63 to −0.11)	150
2022	Semen parameters	Human (n=90)	Experimental study	Self-reported smoking Heavy smokers (>20 cigarettes/day for over 1 year)	Smoking cessation for 3 months was associated with a significant increase in semen volume (2.48±0.79 ml versus 2.90±0.77 ml, $P=0.002$), sperm concentration (18.45×10^{6}/ml±8.56 versus 22.64×10^{6}/ml±11.69, $P=0.001$), and total sperm count ($45.04\pm24.38\times10^{6}$ versus $65.1\pm34.9\times10^{6}$, $P<0.001$)	151
2013	Semen parameters	Human (n=10)	Experimental study	Spermatozoa were exposed to nicotine (0, 1, 10 and 100 ng/ml) over 3 and 24 h	Nicotine decreased sperm progressive motility, reduced spermatozoa viability and increased levels of late apoptosis, altered chromatin compaction and DNA fragmentation in a concentration-dependent manner	152
2019	Semen parameters	Human (n=340)	Observational study	Self-reported smoking ≥1 cigarette/day for >10 years	Infertile smokers showed significant increases in sperm DNA fragmentation ($P<0.001$) and abnormal sperm morphology ($P<0.001$), and significant decreases in sperm counts and motility ($P<0.001$) compared with fertile and infertile non-smokers	154
2012	Semen parameters	Human (n=160)	Observational study	Self-reported smoking	Compared with fertile non-smokers, fertile smokers showed significant increases in sperm DNA fragmentation (10.85±2.37 versus 5.86±1.38) and seminal reactive oxygen species 1,180.7±633.18 versus 436.5±270.7)	155

Table 4 (continued) | Effect of nicotine on reproductive health and offspring outcomes

Year	Primary measure	Species	Study design	Conditions	Results	Ref.
Reproductive health (continued)						
2009	Semen parameters	Human (*n*=13)	Observational study	Cigarette smoke extract (nicotine concentrations from 10 to 100 μg/ml) at 3 and 24 h	Cigarette smoke extract negatively affects sperm motility and chromatin integrity in a dose-dependent and time-dependent manner	156
2021	Testicular volume	Mouse	Experimental study	Nicotine (0.6 mg/kg) or saline for 14 days	Nicotine exposure induces testicular toxicity; it is associated with significantly decreased testicular weight (*P* < 0.05)	157
2017	Testicular volume	Rat (*n*=36 total, 12 control, 12 nicotine at 0.2 mg/kg, and 12 at nicotine 0.4 mg/kg	Experimental study	Nicotine (2 mg/kg or 4 mg/kg) or saline for 7 weeks	Nicotine exposure results in decreased testicular weight (2.210 g ± 0.14 versus 1.89 g ± 0.032, *P* < 0.05) and relative testes to body weight (0.98% ± 0.52 versus 0.72% ± 0.30, *P* < 0.05) compared with controls	158
2012	Erectile dysfunction and sexual function	Human (*n*=2,686)	Cross-sectional study	Self-reported smoking	Heavy smokers (≥20 cigarettes/day) displayed a significantly increased risk of erectile dysfunction compared with never smokers (OR 1.23, 95% CI 1.03–1.49; *P*=0.02). Risk of erectile dysfunction was significantly increased in men smoking >23 years than never smokers (OR 1.60, 95% CI 1.22–2.09; *P*=0.001) Comorbidities and lifestyle factors increased the association between smoking and erectile dysfunction risk: drinking alcohol (OR 1.32, 95% CI 1.01–1.74), physical inactivity (OR 1.33, 95% CI 1.05–1.67), history of hypertension (OR 1.71, 95% CI 1.11–2.62), dyslipidaemia (OR 1.39, 95% CI 1.06–1.81) and diabetes (OR 2.69, 95% CI 1.4–6.98)	163
2005	Erectile dysfunction and sexual function	Human (*n*=2,115)	Population-based study	Self-reported smoking	Current smokers in their 40s displayed the highest odds of erectile dysfunction (OR 2.74, 95% CI 0.44–16.89) compared with men in their 50s (OR 1.38, 95% CI 0.51–3.74), 60s (OR 1.70, 95% CI 0.82–3.51), and 70s (OR 0.77, 95% CI 0.27–2.21)	164
2006	Erectile dysfunction and sexual function	Human (*n*=8,367)	Cross-sectional study	Self-reported smoking	The aOR for erectile dysfunction in smokers was 1.24 (95% CI 1.01–1.52; *P*=0.04) for those smoking ≤20 cigarettes/day and 1.39 (95% CI 1.05–1.83; *P*=0.02) for those smoking >20 cigarettes/day	165
2005	Erectile dysfunction and sexual function	Human (*n*=16,724)	Observational study	Self-reported smoking	The risk of erectile dysfunction was higher in current smokers (≥10 cigarettes/day; aaOR 1.4, 95% CI 1.2–1.5; *P* < 0.0001) and former smokers (aaOR 1.3, 95% CI 1.2–1.5; *P* < 0.0001) than never smokers	166
2004	Erectile dysfunction and sexual function	Human (*n*=2,837)	Observational study	Self-reported smoking	Cessation of smoking for 1 year improves erectile dysfunction more than 25%, but improvement is less in older men. 19 men aged 30–39 years (38%), 9 men aged 40–49 years (27%) and 2 men aged 50–60 years (8%) had improved erectile dysfunction with smoking cessation for 1 year	168
2022	Erectile dysfunction and sexual function	Human (*n*=6,754)	Secondary analysis of a double-blind randomized control trial	Self-reported smoking	Compared with non-smokers, current smokers displayed greater mean total testosterone levels (485.4 versus 451.2 nmol/l, *P* < 0.001) and increased occurrence of low libido (25.6% versus 21.0%; OR 1.34, 95% CI 1.13–1.58; *P*=0.001) and erectile dysfunction (31.6% versus 26.0%; OR 1.43, 95% CI 1.22–1.68; *P*=0.001) with comparable sexual activity (81.7% versus 82.8%, *P*=0.082) Compared with current smokers, former smokers had significantly reduced total testosterone (440.6 vs 485.4 nmol/l, *P* < 0.001) and reduced occurrence of low libido (OR 0.80, 95% CI 0.68–0.96; *P*=0.013) and erectile dysfunction (OR 0.80, 95% CI 0.67–0.94; *P*=0.006)	169

Table 4 (continued) | Effect of nicotine on reproductive health and offspring outcomes

Year	Primary measure	Species	Study design	Conditions	Results	Ref.
Reproductive health (continued)						
2006	Erectile dysfunction and sexual function	Human ($n=819$)	Cross-sectional study	Self-reported smoking	Current smokers who smoked >20 cigarettes/day displayed increased dissatisfaction, erection difficulty and erectile dysfunction compared with never smokers, which increased significantly with age ($P<0.05$; aaOR 1.47, 95% CI 1.00–2.16)	170
Offspring outcomes						
2021	Short-term offspring outcomes	Human (meta-analysis of 8 studies)	Systematic review and meta-analysis study	Self-reported smoking	Paternal smoking of >10 cigarettes/day before conception increased the risk of pregnancy loss (pooled risk estimates; 1.12, 95% CI 1.08–1.16 for 11–19 cigarettes/day; 1.23, 95% CI 1.17–1.29 for ≥20 cigarettes/day)	222
2020	Short-term offspring outcomes	Human ($n=566{,}439$ couples)	Prospective, population-based study	Self-reported smoking	Increased risk of birth defects was found in offspring of fathers who continued smoking (OR 1.87, 95% CI 1.36–2.56; $P<.001$) and decreased smoking (OR 1.41, 95% CI 1.10–1.82; $P=0.007$). Reduced risk of congenital heart diseases, limb abnormalities, digestive tract anomalies and neural tube defects were found in infants whose fathers stopped (OR 0.32, 95% CI 0.15–0.67; $P=0.003$) or decreased smoking (OR 0.25, 95% CI 0.13–0.49; $P=0.000$) before conception	223
2019	Short-term and long-term offspring outcomes	Rat (Sprague–Dawley, $n=18$ breeding pairs total, 9 pairs for the controls and 9 for the treatment group)	Experimental study	0 or 2 mg/kg/day nicotine for 56 consecutive days	Paternal nicotine exposure had no effect on offspring viability, health or growth, but chronic paternal nicotine exposure was linked to altered offspring behaviour, locomotor hyperactivity and impaired habituation	225
2022	Long-term offspring outcomes	Human ($n=48$) and mouse ($n=20$ male offspring in the treatment group, $n=15$ male offspring in the control group)	Observational study (human); experimental study (mouse)	Self-reported smoking (human); water containing 2 mg/ml of cigarette smoke extract (mouse)	Tobacco smoking was associated with significantly increased sperm DNA-methylation changes (2.44±0.16 versus 1.94±0.16), especially hypermethylation in the *DLK1* locus ($P<0.01$) in normozoospermic non-smokers compared with normozoospermic smokers Cigarette smoking extract was associated with significantly increased global DNA methylation levels in spermatozoa and an increased risk of long-term metabolic dysfunction in F1 offspring. In offspring, differentially methylated regions of *Dlk1*, significantly increased *Dlk1* expression in their livers, significantly increased glucose levels ($P<0.001$), and significant reduced LDL ($P<0.01$). An increased accumulation of liver fat was also observed ($P<0.05$)	226
2021	Long-term offspring outcomes	Mouse ($n=10$–12 animals per group)	Experimental study	0.9% sterile saline or nicotine hydrogen tartrate salt (12.6 mg/kg/day, free base weight, dissolved in 0.9% sterile saline) for 28 days	Paternal nicotine exposure enhances fear memory, reduces nicotine administration and alters hippocampal genetic and neural function in F1 and F2 offspring	229
2021	Long-term offspring outcomes	Mouse (cohort A nicotine sired $n=15$; saline sired $n=20$; cohort B nicotine sired $n=7$; saline sired $n=8$)	Experimental study	Nicotine (12.6 mg/kg/day) or 0.9% saline for 28 days	Parental nicotine exposure was associated with decreased risk of nicotine addiction-related phenotypes in offspring	230
2018	Long-term offspring outcomes	Mouse (n values varied between assay but overall ranged from F1 water males $n=3$–18; F1 nicotine male $n=4$–12; for F2 studies: F2 water male $n=10$; F2 female-derived nicotine male $n=11$; F2 male-derived nicotine male $n=5$; F2 water female $n=9$; F2 female-derived nicotine female $n=9$; F2 male-derived nicotine female $n=5$)	Experimental study	Nicotine (200 μg/ml in drinking water) for 12 weeks	Paternal nicotine exposure produces behavioural changes in F1 and F2 offspring, and is linked to nicotine-induced changes to paternal spermatozoa DNA methylation	231

Table 4 (continued) | Effect of nicotine on reproductive health and offspring outcomes

Year	Primary measure	Species	Study design	Conditions	Results	Ref.
Offspring outcomes (continued)						
2012	Long-term offspring outcomes	Human (*n*=39)	Observational study	Newborn umbilical cord blood and maternal peripheral blood	Paternal preconception smoking (% tail DNA: $P > 0.032$; yH2AX foci: $P > 0.018$) induced DNA damage in the F1 offspring cord blood	232
2013	Long-term offspring outcomes	Human (*n*=295)	Observational study	Self-reported smoking	In offspring of non-smoking mothers, paternal smoking was associated with 46% (95% CI 21–64%) reduced total sperm count	234

aaOR, age-adjusted odds ratio; aOR, adjusted odds ratio; MD, mean difference; OR, odds ratio.

normal sperm morphology (MD 1.37%, 95% CI 2.63–0.11, $P = 0.03$)[150]. Evidence has shown a deleterious effect of nicotine and tobacco smoke exposure on semen parameters, but a few studies have examined the potential benefits of smoking cessation. In a 2022 study, semen parameters in 48 men (aged 28–41 years) who smoked an average of 30 cigarettes per day before and after smoking cessation for 3 months were compared, and significant increases in semen volume (2.48 ml ± 0.79 ml versus 2.90 ml ± 0.77 ml, $P = 0.002$), sperm concentration (18.45×10^6/ml ± 8.56 versus 22.64×10^6/ml ± 11.69, $P = 0.001$) and total sperm count (45.04×10^6/ml ± 24.38 versus 65.1×10^6/ml ± 34.9, $P < 0.001$) were found, with positive yet insignificant trends in sperm motility (20.54% ± 15.72 versus 21.41 ± 14.97, $P = 0.190$) and sperm morphology (2.22% ± 1.69 versus 2.43 ± 1.47, $P = 0.120$)[151]. In isolated spermatozoa from 10 non-smoking men with normozoospermia, results of a previous study showed that in vitro nicotine exposure suppressed sperm motility in a dose-dependent manner, even when the lowest concentration was used (1 ng/ml, $P < 0.05$)[152,156]. The results of this study demonstrated that nicotine, the main component of cigarette smoke, can independently alter sperm motility in a dose-dependent manner, so counselling patients who are unable to or unwilling to quit smoking regarding the benefits of limiting nicotine exposure is important.

In general, evidence suggests that nicotine use adversely affects semen characteristics, potentially in a dose-dependent manner. However, the mixed results reported are likely a result of confounding factors, including combustible products from tobacco smoke, the type and duration of use, and the time at which semen samples were collected. Future studies should include the type, frequency and duration of tobacco products used, and attempt to coordinate semen collections at a similar time of day across participants.

Testicular volume. Studies assessing the effect of nicotine on testicular volume are preliminary in nature; however, two studies in both mice and rats suggest that nicotine administration significantly reduces total testicular weight relative to total body weight[157,158]. In the mice, nicotine was injected intraperitoneally (0.6 mg/kg) for 14 days and nicotine decreased testicular weight significantly ($P < 0.05$)[157]. Similarly, rats were injected intraperitoneally with saline (0.2 ml) or high-dose (0.4 mg/kg) nicotine and notable decreases in testicular weight (2.210 g ± 0.14 versus 1.89 g ± 0.032, $P < 0.05$) and relative testes-to-body weight (0.98% ± 0.52 versus 0.72% ± 0.30, $P < 0.05$) were observed[158]. These findings are concerning; at this time, data are insufficient to conclude that nicotine use negatively influences testicular volume and further animal studies with strong translational relevance, recapitulating typical human use and mimicking the male HPG axis, are needed.

Erectile dysfunction and sexual function. The use of nicotine and cigarettes has been linked to erectile dysfunction, but many men are unaware of this risk[159,160]. The underlying aetiology is largely thought to be secondary to vascular mechanisms[160], primarily depletion of nitric oxide (an important neurotransmitter found in cigarette smoke[161]) that regulates penile vessel constriction and relaxation of the corpora cavernosa to achieve penile erection[162]. Men with vascular risk factors such as hypertension or diabetes and who smoke are at increased odds of developing erectile dysfunction[160].

In a cross-sectional study, ~23% of instances of erectile dysfunction were associated with cigarette smoking[163]. In this study of 2,686 men (22.9% smoked <20 cigarettes per day and 31.5% smoked ≥20 cigarettes per day), individuals who smoked for >23 years had a significantly higher risk of erectile dysfunction than never smokers (OR 1.23, 95% CI 1.03–1.49, $P = 0.02$). In another population-based study including 1,329 men with a regular sexual partner (173 current smokers and 836 had previously smoked) in which former and never smokers were compared, the risk of erectile dysfunction was highest among current smokers in their 40s (OR 2.74, 95% CI 0.44–16.89, $P < 0.09$)[164]. In addition, non-smoking men were significantly less likely to experience erectile dysfunction than men who were current (OR 1.17, 95% CI 0.71–1.94, $P < 0.09$) or ever smokers (OR 1.46, 95% CI 1.05–2.02, $P < 0.09$)[164]. Results of a cross-sectional study of 8,367 men (8.8% non-smokers, 9.4% ≤20 cigarettes per day, 14.7% >20 cigarettes per day) also reported that smoking was significantly associated with the occurrence of erectile dysfunction following a dose-dependent pattern[165]. Compared with non-smokers, the adjusted odds ratio for erectile dysfunction was 1.24 (95% CI 1.01–1.52, $P = 0.04$) in men smoking ≤20 cigarettes per day and 1.39 (95% CI 1.05–1.83, $P = 0.02$) for those smoking >20 cigarettes per day. Results of similar studies also demonstrated a positive association between smoking frequency/duration and erectile dysfunction[166,167]. Some evidence shows that smoking cessation improves erectile dysfunction rates, but is mostly limited to men under the age of 50 years lacking comorbidities and with minor smoking histories[160]. In a prospective study of 2,837 smokers (aged 30–60 years), 637 (22.5%) reported having erectile dysfunction, which correlated with the level of exposure to smoking[168]. Of the 118 patients who stopped smoking, erectile dysfunction improved after a year in >25%; however, older individuals experienced less improvement[168]. Overall, 19 of men aged 30–39 years (38%), 9 of men aged 40–49 years (27%), and 2 of men aged 50–60 years (8%) had improved erectile dysfunction with smoking cessation for 1 year[168].

Sexual function increased following smoking cessation in 6,754 men aged 50–75 years and grouped into non-smokers (3,069; 45.4%), former smokers (2,673; 39.6%) and current smokers (1,012; 15%)[169]. Current smokers had a higher prevalence of low libido than former and non-smokers (25.6% versus 21.0%, $P = 0.002$) with comparable sexual activity (81.7% versus 82.8%, $P = 0.420$)[169]. Cigarette smoking is associated with worse sexual health than in non-smokers; the decrease in libido was not enough to reduce the frequency of sexual activity. In 819 men aged 31–60 years, current smokers who smoked ≥20 cigarettes had greater sexual dissatisfaction than never smokers[170]. These findings highlight cigarette smoking as a modifiable lifestyle behaviour in men with sexual dissatisfaction and dysfunction.

Overall, nicotine use is associated with erectile dysfunction and decreased sexual function, including low libido and sexual satisfaction. These outcomes seem to be dose dependent, based on the number of cigarettes smoked or amount of tobacco product used per day, and are at increased likelihood in men with underlying vascular conditions, such as diabetes, than in those without. Thus, counselling patients undergoing evaluation for sexual dysfunction to cut back or abstain from nicotine use is important.

Summary. Nicotine use is highly prevalent and evidence largely suggests that it negatively affects male reproductive health, especially semen parameters and sexual function (Table 4). The availability of different tobacco products and inconsistent adjustment of confounders, including polysubstance use, contribute to mixed findings. Health-care providers must discuss with patients that nicotine use could adversely affect male fertility and the benefits of cessation or limited use.

Paternal health and behavioural lifestyles, such as substance use, can affect male reproductive and offspring outcomes. Substance use, especially among reproductive-age men, continues to be an ongoing issue partly owing to the lack of awareness regarding the potential influence on reproductive health. Research has shown that substance use can affect semen parameters, male reproductive hormone secretion and sexual function. Thus, identifying these modifiable lifestyle paternal factors with regard to reproductive outcomes is important. This intervention is especially urgent considering the growing evidence highlighting that preconception paternal substance use is linked to adverse offspring development, including congenital anomalies, low birthweight, and metabolic and neurodevelopmental disorders[10,11,171–173].

Offspring outcomes

Accumulating evidence suggests that preconception paternal substance use can result in adverse consequences for offspring, including abnormal brain development, neurobehavioural dysfunction and worsened mental health[10]. Increased offspring morbidity can occur both in the short term (that is, the neonatal period and infancy) in addition to the long term (that is, childhood through to adulthood). The most common substances used include alcohol, cannabis, opioids and nicotine.

Alcohol

Paternal alcohol consumption is associated with negative influences on offspring growth, development, neurodevelopment and sociobehaviour that might have a greater effect on boys than on girls[5,174–178] (Table 1).

Short-term outcomes. In animal studies, preconception paternal alcohol consumption has been linked to intrauterine growth restriction[5,174], craniofacial growth deficiencies[175], low birthweight[5,174], limited growth[5] and altered reproductive development[179] in offspring. Affected growth patterns have been reported as sex specific, with an increased effect on male offspring[174]. In male mice, preconception paternal alcohol exposure (10% ethanol for 4 h daily) for 70 days before mating was associated with a significantly increased incidence of intrauterine growth restriction (25% reduction in body weight, $P = 0.004$) and male offspring were more greatly affected than females (15% reduction in body weight, $P = 0.017$)[174]. In another study of adult male mice exposed daily to 10% ethanol (2.7 g/kg), 2D imaging on collected fetal heads post-delivery demonstrated that preconception male alcohol exposure induces a significant increase in craniofacial growth deficiencies in offspring ($P < 0.0001$)[175]. The results of these studies suggest that paternal alcohol use before conception can affect offspring development. A prospective cohort study of 1,292 pregnancies through in-person interview demonstrated that offspring in the paternal-exposed group (exposure to alcohol within the 3 months before conception) had a shorter anogenital distance than the unexposed group at birth and at 6 months, especially in boys ($P = 0.01$ and $P = 0.02$, respectively) compared with girls ($P = 0.04$ and $P = 0.04$, respectively)[179]. This observation indicates that paternal alcohol consumption can have an adverse effect on offspring reproductive development. In rats, acute paternal alcohol exposure (intraperitoneal injection with alcohol 5 g/kg) compared with saline exposure 24 h before breeding resulted in a ~50% reduction in viable offspring[180]. These results suggest that even acute paternal alcohol use can adversely affect fertility and fetal outcomes. A large population study involving 529,090 couples from the National Free Preconception Health Examination Project determined that the risk of birth defects was increased with paternal alcohol consumption (drinking at least once per week) (OR 1.35, 95% CI 1.14–1.59, $P < 0.001$), especially the risk of cleft lip or palate (OR 1.55, 95% CI 1.04–2.30, $P = 0.03$)[176]. This study only reported the frequency, but not the amount, of alcohol consumed but it suggests that future fathers should be counselled to modify their alcohol intake before conceiving.

Long-term outcomes. Preclinical studies have shown that preconception paternal alcohol consumption can alter the sperm genome and epigenome, including changes in DNA methylation[181], chromatin structure modifications[182] and small non-coding RNAs,[183]. In a study of adult mice exposed to vapour ethanol or room air (8 h a day for 5 days per week) over 5 weeks, significantly altered expression of several small non-coding RNA species ($P < 0.05$) in sperm was reported. As small non-coding RNAs in sperm can induce heritable phenotypes in offspring, these findings suggest a potential underlying mechanism for how paternal alcohol consumption might affect offspring development and health[183]. Results of another study of male mice showed that exposure to 10% ethanol compared with saline ad lib for 35 days can significantly disturb sperm DNA integrity and chromatin remodelling ($P < 0.05$)[182]. These changes could contribute to abnormal offspring neurodevelopment including anxiety, depression, cognitive impairment and ADHD[177,178,184]. In the prospective Shanghai-Minhang Birth Cohort Study of 796 children, preconception paternal alcohol consumption was associated with an increased risk of anxiety and depression in male offspring at age 4 years (RR 1.33, 95% CI 1.09–1.61) and 6 years (RR 1.37, 95% CI 1.02–1.85)[184]. In a study of male mice ($n = 15$) injected intraperitoneally with 20% ethanol (5 mg/kg) compared with saline before mating, alcohol-sired pups had delayed motor milestones ($P < 0.05$), decreased frequency of risk assessment behaviour ($P < 0.001$), increased aggressive behaviours ($P < 0.001$) and increased defensive behaviours ($P < 0.0001$)[178]. Results of a study of mice exposed to ethanol via oral gavage (3.3 g/kg ethanol or 1.1 g/kg ethanol) demonstrated that offspring of paternally ethanol-exposed mice had greater anxiety-like behaviour, and decreased learning ability and memory ($P < 0.05$), than mice that received saline for 1 month[177].

Similarly, a link between paternal drinking and an increased incidence of externalizing psychopathological disorders[5,185], congenital heart defects[186] and cancer (such as leukaemia, brain tumours and neuroblastoma)[187] in children. Data from the Minnesota Twin Family

Study, a community-based investigation through structured interviews of adolescents (age 17 years, $n = 1{,}252$) and their parents, demonstrated that parental alcohol dependence (both or one parent) was associated with an increased risk of externalizing psychopathology in late-adolescent offspring, including ADHD (OR 2.77, 95% CI 1.18–6.46, $P < 0.05$), oppositional defiant disorder (OR 2.28, 95% CI 1.46–3.56, $P < 0.001$), conduct disorder (OR 1.85, 95% CI 1.27–2.68, $P < 0.01$), adult antisocial behaviour (OR 2.25, 95% CI 1.22–4.14, $P < 0.01$), nicotine dependence (OR 1.96, 95% CI 1.27–3.05, $P < 0.01$), alcohol dependence (OR 2.18, 95% CI 1.32–3.61, $P < 0.01$) and drug dependence (OR 2.25, 95% CI 1.09–4.62, $P < 0.05$)[185].

A population-based case–control study using data from the Guangdong Registry of Congenital Heart Disease, an ongoing WHO population-based surveillance system, showed that paternal alcohol consumption was associated with an increased risk of congenital heart disease (aOR 2.87, 95% CI 2.25–3.65)[186]. The most common congenital heart defects associated with paternal alcohol consumption were left ventricular outflow tract obstruction (12.7%), atrial septal defect (9.1%), tetralogy of Fallot (6.9%) and transposition of the great arteries (6.7%)[186]. In addition, results of a prospective cohort study including 4,710 men (aged 18–20 years) demonstrated that paternal alcohol consumption, even in moderate amounts (20–60 g of alcohol consumed per week), is linked to an increased risk of substance-related disorders in their children. Paternal volume of alcohol consumed was associated with the risk of offspring substance-related disorders; the risk ranged from HR 1.11 (95% CI 0.84–1.45) for the lowest drinking quintile (up to 20 g alcohol per week) to HR 2.02 (95% CI 1.56–2.62) for the highest drinking quintile (80–100 g alcohol per week)[188]. These observations suggest that paternal alcohol consumption, especially quantity consumed, can influence offspring risk of addiction.

Summary. In general, results preclinical and human studies have shown that preconception paternal alcohol consumption can adversely affect offspring development and function (Table 1). Prenatal alcohol exposure from maternal use receives increased focus, but counselling men interested in conceiving and recommending cessation of paternal alcohol consumption before conception is equally important.

Cannabis

Paternal cannabis use has been linked to adverse offspring outcomes including increased miscarriage rates and altered neurobehavioural outcomes such as hyperactivity and poor attention[189,190] (Table 2). In preclinical studies, cannabis exposure has also been inconsistently associated with congenital anomalies[191].

Short-term outcomes. The effects of cannabis use on offspring outcomes have been most frequently studied in relation to maternal exposure, but growing evidence suggests that paternal exposure might also have deleterious effects. Paternal cannabis use and perinatal outcomes in mice and rats have been the focus of several studies. Impairments in placental development and offspring growth were noted in those with sires exposed to a CB2R agonist for 5 weeks[192]. Additionally, offspring from male mice exposed to THC (50 mg/kg) orally three times a day for 5 weeks were significantly more likely to be lost during pregnancy (37% versus 19%, $P < 0.05$)[193] than those given oil, and the sperm from these mice was found to have an increased prevalence of chromosomal abnormalities such as aneuploidy (9.66 versus 0.27, $P < 0.05$), polyploidy (5.4 versus 3.88, $P < 0.05$), and translocation events (0.54 versus 3.12, $P < 0.05$)[193]. A dose of 50 mg/kg THC in mice corresponds to an oral dose of approximately 4 mg/kg in humans, equivalent to three cannabis cigarettes containing 1% THC[193]. However, in another mouse study using a similar duration of THC exposure (every other day for 5 weeks), but a much lower THC dose (10 mg/kg versus 50 mg/kg body weight), was not able to recapitulate the findings of fetal loss[116]. These findings suggest a dose-related effect with considerably adverse offspring outcomes most associated with heavy, daily cannabis use before conception.

In human populations, increased paternal use of cannabis preconception has been linked to decreased infant birthweight in couples undergoing in vitro fertilization[194]. Results of a prospective study including 221 couples undergoing in vitro fertilization showed that men who used cannabis a year before the procedure had 1 less embryo transferred (95% CI −1.25 to −1.02, $P = 0.04$)[194]. In addition, the lifetime amount of cannabis smoked before in vitro fertilization was associated with decreased infant birthweight, moderate use (11–90 times) was associated with a 15% decrease (95% CI −0.3 to −0.01, $P = 0.03$) and heavy use (>90 times) was associated with a 23% decrease (95% CI −0.46 to −0.07, $P = 0.01$) in infant birthweight[194]. By contrast, no association was found in a large cohort study of 2,642 men (115 cannabis users and 2,527 non-users) between paternal cannabis use and changes in birthweight (40.68; 95% CI −56.71 to 138.06, $P = 0.41$), but only paternal cannabis usage during pregnancy rather than the preconception period was examined[195]. These results suggest that the timing of paternal cannabis use, especially before conception, can influence the potential for adverse offspring outcomes. Regarding fetal loss, a large prospective study of 1,535 couples (9% reported male cannabis use less than once a week and 8% reported cannabis use at least once a week, with half using daily) conducted online found that couples in which the man used cannabis at least once per week were at an increased risk of experiencing spontaneous abortion (hazard ratio 2, 95% CI 1.2–3.1)[196]. Associations between paternal cannabis usage before conception, during pregnancy, and postnatally with sudden infant death syndrome (SIDS) have also been reported in a case–control study[197]. In a study involving 239 infants who died of SIDS matched to 239 healthy infants, an increased risk of SIDS was associated with fathers who used cannabis during conception (OR 2.2, 95% CI 1.2–4.2, $P = 0.01$), during pregnancy (OR 2, 95% CI 1.0–4.1, $P = 0.05$) and postnatally (OR 2.8, 95% CI 1.1–7.3, $P = 0.04$)[197]. The findings of this study underscore the role of paternal substance use, specifically the relationship between cannabis use and SIDS.

Long-term outcomes. Evidence from animal studies has largely highlighted that paternal cannabis use might influence offspring development and behaviour. In a rat model, paternal exposure to THC (0 or 2 mg/kg/day oral gavage) for 12 days was associated with significantly reduced attentional performance ($P < 0.025$)[198]. In another study in male rats, THC (0, 2 or 4 mg/kg/day subcutaneously) for 28 days adversely affects offspring behavioural effects, including increased locomotor hyperactivity in adolescent and adult offspring ($P < 0.05$)[199]. These findings support the influence of paternal cannabis use on offspring development, particularly behaviour. Corresponding data in humans are comparatively limited, but associations between paternal cannabis use and offspring behavioural problems have been described[200]. A population-based birth cohort study ($n = 5{,}903$) noted that paternal cannabis use, through smoking, reported by questionnaire, was associated with child externalizing problems by 7–10 years old (B = 0.36, 95% CI 0.22–0.49) but not internalizing problems measured by validated teacher, child and mother reports. Externalizing behaviour has been shown to have common genetic and environmental origins[201] and although the literature often focuses on maternal contributions, these reported observations reflect the important role of paternal contributions to offspring development[200].

Paternal cannabis use has also been inconsistently linked to offspring congenital anomalies. In a study of rats, offspring born to cannabis extract-exposed fathers (intraperitoneal injection of 4 mg/kg/day THC) exhibited significant rates of cardiomegaly relative to those born to control fathers ($P = 0.0013$)[202]. These cardiac findings in offspring were associated with altered sperm DNA methylation of genes associated with early developmental processes in the cannabis extract-exposed father[202]. This observation supports the theory of paternal origins of health and disease, in which paternal environmental exposures can disrupt early offspring development and the importance of healthcare provider counselling for individuals interested in conceiving. Furthermore, results of several studies based on the Baltimore-Washington Infant Study, a regional epidemiological study of congenital heart disease, and the Atlanta Birth Defects Case–Control Study showed associations between paternal cannabis use with transposition of the great arteries (RR 1.4, 95% CI 2.8–12.7, $P = 0.05$)[203], ventricular septal defects (OR 1.36, 95% CI 1.05–1.76)[204] and single-ventricle defect (OR 2.2, 95% CI 1.0–5.2)[205,206]. Taken together, these findings highlight the need to consider paternal environmental factors in the pathogenesis of fetal cardiac abnormalities and the importance of preconception counselling. In addition, an increased risk of rhabdomyosarcoma by two-fold (95% CI 1.3–3.3) in the child has also been associated with the paternal use of cannabis during the year before their child's birth[207]. This observation suggests that paternal cannabis use before conception can increase offspring risk of rhabdomyosarcoma and that lifestyle modification might be beneficial in reproductive-age men to reduce offspring morbidity.

The underlying mechanisms by which paternal cannabis exposure might influence offspring outcomes are not fully understood; emerging evidence suggests that epigenetics, including DNA methylation, might have a role in the transmission of outcomes[10]. In recreational cannabis users, widespread alterations in sperm DNA methylation were found compared with non-users[80], including hypomethylation of *DLGAP2* (ref. 208), a gene that has been associated with or implicated in the pathogenesis of autism spectrum disorders[208,209]. Sperm from male rats exposed to cannabis demonstrated changes in methylation that were also found to be present in their offspring, suggesting the potential for transmissibility[202]. Similarly, alterations in DNA methylation patterns noted in mouse sperm exposed to synthetic cannabinoids were transmitted to placental tissues, with some of the affected genes known to be involved in placental and embryonic development[192]. The importance of these findings is that paternal selective activation of cannabinoid receptors in the sperm epigenome can affect pregnancy outcomes. Male cannabis users ($n = 30$) had increased incidence of hypermethylation in *DRD2* and *NCAM1*, genes that are involved in dopaminergic pathways and that have been associated with substance use disorders, including nicotine and alcohol dependence[210–212]. However, whether these changes are transmissible to offspring in a clinically meaningful way has yet to be demonstrated and will require further investigation. If transmissible, concern for increased offspring addiction risk would exist and preventative interventions would be beneficial, including early referral to support services.

Summary. Preclinical and human studies suggest that paternal cannabis use might increase pregnancy loss and can adversely affect short-term and long-term offspring outcomes, including development and behaviour (Table 2). The underlying mechanism for these observations is not well understood but could be a result of inherited epigenetic alterations. Additional research in relevant animal models is needed to overcome the limitations and confounding variables of human studies, to better examine the relationship between paternal cannabis use, including dose, frequency and duration used, and offspring development and outcomes.

Opioids

Research on the effect of paternal opioid use on offspring health outcomes is limited, especially regarding a dose-related effect. Dose–response studies would provide information regarding the safest effective dose. Existing evidence suggests that it is associated with changes in offspring weight, neurobehaviour, pain perception and vulnerability to opioid abuse[172,213–216] (Table 3). In preclinical studies, offspring with fathers who used opioids, especially males, demonstrated increased withdrawal-like behaviours, sensitivity to the antinociceptive properties of opioids, delayed learning and impulsivity[172,214–216].

Short-term outcomes. Little is known about the effect of chronic opioid exposure on the next generation, let alone from paternal preconception opioid use. Much of the existing research on the transgenerational influence of paternal opioid use has been studied in conjunction with maternal opioid exposure[10]. Existing evidence on paternal preconception opioid use suggests an influence on fetal organ weights (such as adrenal gland and thymus)[217] and offspring hormone levels (including testosterone)[218] that are involved in growth-regulation and neurotransmitter function[171]. A study of male rats subcutaneously injected with methadone (5 mg/kg) for 4 days before undergoing mating compared with untreated males had offspring notable for significantly heavier adrenal glands (29.02 mg ± 0.81 versus 23.75 mg ± 1.05, $P < 0.05$) and lighter thymus glands (65.37 mg ± 4.18 versus 72.56 mg ± 2.26, $P < 0.05$)[217]. Results of another study of male rats implanted with morphine pellets before conception were notable for offspring with significantly decreased serum testosterone levels (3.18 ng/ml ± 0.27 versus 4.37 ± 0.37, $P < 0.01$)[218]. These results indicate that paternal opioid use can affect offspring development and endocrine function. In a study involving 8,410 parent–offspring pairs, regular preconception paternal opioid use was independently associated with increased odds of offspring being overweight (adjusted OR (aOR) 1.76, 95% CI 1.15–2.71)[213]. These observations suggest that paternal opioid use before conception can influence offspring metabolic health and given the obesity pandemic, suggests that opioid use is a modifiable lifestyle factor to consider.

Long-term outcomes. Emerging evidence shows that preconception paternal opioid exposure might influence developmental trajectories across multiple generations[171,213,218,219]. Preclinical studies suggest that paternal opioid use can affect offspring behaviour and neurobiological characteristics, including increased withdrawal-like behaviours, synaptic plasticity deficits and altered sensitivity to the antinociceptive properties of opioids[172]. In addition, findings suggest that paternal opioid use might increase offspring vulnerability to opioid abuse in a sex-dependent manner, with males often more affected[172]. In a study of male rats injected with morphine (25 mg/kg) the day before mating, male offspring of morphine-exposed fathers had a significantly enhanced sensitivity to the antinociceptive effects of morphine ($P < 0.01$) compared with male offspring of unexposed sires; however, no significant effect was seen in female offspring[219]. In another study of male rats injected with morphine twice daily (10 mg/kg every 12 h) for 2 weeks, significantly higher anxiety-like behaviour and enhanced voluntary consumption of morphine in male offspring of morphine-exposed fathers than in those whose fathers were exposed to saline ($P < 0.01$)[220]. These findings are concerning and support the influence of paternal opioid use on offspring development, with the greatest effect on male offspring.

In a rat model, paternal preconception exposure to opioids has been shown to increase sensitivity to the pain-relieving effects of morphine in male offspring[221]. An intergenerational effect of paternal morphine exposure during adolescence on pain perception and the antinociceptive effect of morphine in rat offspring have also been demonstrated[214]. In adolescent male rats receiving subcutaneous morphine (2.5 mg/kg initially and increased to 25 mg/kg over 40 days) compared with saline before mating, offspring had significantly decreased pain-related behaviours and an antinociception effect ($P < 0.001$)[214]. A paternal opioid self-administration rat model (0.75 mg/kg/infusion of morphine over 5 s for 3 h a day) to mimic the human condition of voluntary consumption showed that paternal morphine exposure compared with saline-exposed controls selectively disrupts novel object recognition in female ($P = 0.011$) but not male ($P = 0.965$) progeny[216]. However, anxiety-like behaviour or stress-induced hypothalamic–pituitary–adrenal axis activation was changed in neither male nor female offspring[216]. In another study, male Wistar rats received escalating subcutaneous doses of morphine (2.5–25 mg/kg) or saline for 10 days[215]. Offspring sired by rats that had received morphine exhibited delayed learning ($P = 0.002$) and impulsive behaviour ($P < 0.001$) compared with offspring sired by control rats[215]. Taken together, the findings of these studies suggest that paternal opioid exposure can affect socioemotional behaviour and response to pain in progeny.

Summary. Evidence examining the influence of paternal opioid use on offspring health outcomes is lacking. Results of preclinical and human studies have shown that paternal opioid use is associated with adverse effects on offspring weight, neurodevelopment, sociobehaviour, sensitivity to pain and addiction vulnerability, with male offspring more likely to be affected than female offspring (Table 3). The social consequences of paternal opioid use can result in an increased prevalence of mental health and addiction disorders, which can be associated with increased morbidity and mortality. More research is needed to uncover the mechanisms underlying these observations so that potential intervention or targeted therapies can be developed.

Nicotine

Overall, evidence regarding paternal nicotine product use and pregnancy and offspring outcomes is limited. Paternal nicotine product use has been linked to increased pregnancy loss, birth defects and abnormal offspring neurobehaviour[222,223] (Table 4). Some of these adverse effects seem to be dose dependent and potentially mitigated by a reduction or cessation of paternal nicotine use.

Short-term outcomes. Most research on the intergenerational effects of parental nicotine product use has focused on maternal exposure, with little known about paternal use[224]. Evidence from human studies and animal models has shown a potential link between paternal preconception nicotine exposure and offspring health. In a rat model of chronic nicotine exposure, paternal nicotine exposure did not affect offspring viability, health or growth[225]. By contrast, results of a systematic review and meta-analysis of eight human studies including men and women showed that, after adjusting for maternal smoking status, preconception paternal smoking of >10 cigarettes daily was linked to an increased risk of pregnancy loss in a dose-dependent manner[222]. No adverse effects were found with paternal smoking of 1–10 cigarettes per day. In a prospective, population-based study, pregnancy outcomes of 566,439 couples based on preconception paternal smoking habits were investigated[223]. A significantly higher prevalence of birth defects was observed in children of couples with continued (OR 1.87, 95% CI 1.36–2.56, $P < 0.000$) or reduced (OR 1.41, 95% CI 1.10–1.82, $P = 0.007$) paternal smoking during pregnancy[223]. Interestingly, in the 1:1 case–control (birth defects compared with normal pregnancy) analysis, reduced risks of congenital heart diseases, limb and digestive tract anomalies, and neural tube defects were observed in couples with decreased (OR 0.025, 95% CI 0.13–0.49, $P = 0.000$) or ceased (OR 0.32, 95% CI 0.15–0.67, $P = 0.003$) paternal smoking during pregnancy[223]. These results suggest that changes in smoking behaviour, including cessation or reduction, might reduce the risk of miscarriage and offspring birth defects.

Long-term outcomes. Existing evidence suggests that paternal nicotine exposure can induce epigenetic changes, including alterations in DNA methylation (both hypomethylation and hypermethylation) and histone modification, that might result in offspring neurobehavioural and metabolic effects[226–228]. Results of studies in rats have shown that chronic paternal nicotine exposure can substantially alter offspring behavioural function, including impaired habituation and locomotor hyperactivity[225]. In male rats exposed to saline or 2 mg/kg/day nicotine subcutaneously for 56 days, paternal nicotine exposure was associated with a significant ($P < 0.025$) degree of locomotor hyperactivity in juvenile male offspring (41.1 ± 1.7) compared with male controls (34.5 ± 1.7)[225]. In addition, offspring of nicotine-treated males demonstrated a pattern of slower habituation of locomotor behaviour than those with saline-exposed fathers ($P < 0.05$)[225]. In both humans and a mouse model, paternal exposure to cigarette smoke altered sperm DNA methylation in the F1 generation at genes, including *DLK1*, that are involved in metabolic function[226]. Taken together, the findings of these studies suggest that paternal exposure to cigarette smoke can result in behaviour and epigenetic changes in offspring that might affect long-term outcomes, including metabolic function and behavioural health.

In a C57BL6/J mouse model, paternal nicotine exposure was potentially shown to have a multigenerational effect of increasing offspring susceptibility to anxiety disorders such as post-traumatic stress disorder[229], but might also result in decreased susceptibility to nicotine addiction-related phenotypes[230]. Paternal preconception smoking also resulted in significantly increased spontaneous locomotor activity (10,173 ± 5,371.4 versus 5,655 ± 2,041.99 and 14,571 ± 6,248.4 versus 9,995 ± 4,153.6, $P < 0.05$) and significantly decreased reversal learning (16.41 ± 8.31 versus 8.19 ± 4.87 and 14.05 ± 7.41 versus 4.13 ± 4.46, $P < 0.01$) of F1 male and female mice, respectively, which could relate to the increased occurrence of neurobehavioural disabilities such as ADHD and autism[231]. These findings also highlight the potential differences in sensitivity of male versus female offspring to nicotine exposure. F1 male offspring were also observed to have significantly reduced attention, brain monoamine (for example, dopamine, noradrenaline and metabolites) levels, and dopamine receptor mRNA expression ($P < 0.05$)[231].

Analysing the effects of paternal preconception tobacco smoke exposure on human offspring has been a challenge because the separation of preconception and gestational exposures are difficult to control. Offspring cord blood analysed for genomic stability contained predictors of increased single-strand and double-strand DNA breaks with paternal smoking. Thus, paternal smoking habits could provide a mechanistic basis for genetic instability in offspring and preconception and gestational cigarette smoking could have an intergenerational risk of the induction of DNA damage in human offspring[232]. Spermatozoa DNA damage can increase the likelihood of unsuccessful fertilization, miscarriage, impaired embryo quality and altered embryonic development[233]. The semen parameters of 295 adolescent men showed that those with fathers exposed to cigarette smoke displayed 46% lower total sperm counts and lower sperm concentrations, which might adversely affect their fertility and ability to conceive[234].

Summary. The evidence is conflicting, but overall suggests that paternal nicotine use can negatively affect short-term and long-term offspring health and that adverse outcomes are probably dose dependent (Table 4). Inconsistent adjustment of confounders, including tobacco smoke and maternal nicotine use, probably contributed to the variability in study findings. Future research needs to also focus on understanding the benefits of cessation in addition to the effects of dose, frequency and duration of exposure on offspring outcomes to improve counselling of patients regarding paternal nicotine use.

Public health

The influence of paternal health and behavioural lifestyles, such as substance use, on male fertility and offspring outcomes is an understudied and underappreciated topic in reproductive health. Existing health campaigns by large organizations, including the Centers for Disease Control and Prevention, the March of Dimes, the Substance Abuse and Mental Health Services Administration, National Institutes of Health and the WHO, are focused on improving maternal health; similar measures targeting paternal health and lifestyle are largely unavailable. Few guidelines and recommendations exist to direct health-care providers in discussing substance use with patients intending to conceive owing to limited safety data. The American Society of Reproductive Medicine and the American Urological Association have issued committee opinions to assist practitioners in optimizing fertility, including substance use counselling, but highlight the need for more evidence-supported recommendations[235,236]. Thus, additional research is urgently needed to inform guideline development, prevention campaigns and programme implementation to promote male reproductive health. To achieve this aim, heightened awareness and recognition of the paternal role in achieving successful healthy offspring is needed. Currently, well-established public health policies exist advising future mothers not to consume alcohol, smoke or eat unhealthily to benefit their offspring. However, appreciation by the general population that paternal contributions are evidently relevant and can adversely affect male fertility in addition to prenatal health outcomes is lacking[173]. Increased public understanding regarding the potential adverse effect of paternal substance use on future offspring health will encourage professional and parent-implemented early intervention strategies to help to decrease developmental delays or disabilities and maximize developmental potential[237,238].

Patient counselling

Crucially, health-care providers need to counsel men of reproductive age and men and couples undergoing reproductive decisions regarding the negative effects of substance use on male fertility and offspring development, and the potential benefit of cessation of or reducing substance use. The growing prevalence of substance use can partly be attributed to perceptions of safety because insufficient evidence exists regarding its risks and patient counselling by health-care providers is lacking. To mitigate the potential adverse effects of substance use, health-care providers must engage in evidence-informed discussions with patients about the reproductive health effects of substance use. However, many providers have cited a lack of training, clarity of the evidence on health effects, and limited practice talking to patients about these topics as barriers to discussing substance use. Thus, a crucial need exists to design and disseminate educational strategies and resources to teach clinicians so that they can effectively provide information and appropriate counselling. In addition, professional health-care societies should focus on providing more directive and up-to-date guidelines for practitioners to follow.

Overall, safe limits for consumption are not known so the safest option to avoid harmful effects to fertility and offspring health is cessation. For patients who are not ready to stop their substance use or feel that stopping is not the best option for them, the goal should be harm reduction. Harm-reduction strategies include considering safer alternatives to the substance used, using reduced amounts, and decreasing frequency of use given that a dose-dependent and/or duration-dependent response is often observed. Substance use is an important modifiable lifestyle risk factor that can be optimized before conception to improve fecundity, and pregnancy, birth, and short-term and long-term outcomes in offspring.

Future directions

Additional research is needed to further understand the magnitude of the effects of substance use on male fertility, especially to determine whether a dose-dependent effect exists, and if the changes are reversible or permanent. Also, as the prevalence of the co-use of marijuana or opioids with nicotine and/or alcohol consumption is high[239], preclinical studies need to reflect real-world use and elucidate the effects of each substance individually as well as in combination. Characterizing the role and contributions of paternal origins of disease on short-term and long-term offspring health outcomes is also crucial, including neurodevelopmental and sociobehavioural outcomes. This characterization would highlight the importance of paternal lifestyle, diet and other environmental exposures on future offspring health[173]. Research is also needed to determine the underlying mechanisms of potential intergenerational transmissibility. DNA methylation is one of the most frequently studied means of potential paternal transmission of non-genetic characteristics to offspring, but future work should investigate the role of retained histone proteins and other non-genetic components of epigenetic inheritance because of their influence on key developmental processes[10].

Evidence-based counselling is also needed for individuals or couples intending to conceive, to inform preconception lifestyle modifications. As studies have shown that many health-care providers do not address the safety of cannabis use, in part owing to a lack of training and the clarity of the evidence on health effects[240], a crucial need to educate health-care providers exists so that they can effectively provide information and appropriate counselling to patients using cannabis. Furthermore, rational developmental screening strategies for exposed offspring are needed, as well as personalized therapeutic strategies, including cognitive behavioural therapy and targeted behaviour interventions, to mitigate the potential adverse effects of early environmental risk factors[241].

Conclusions

Substance use has been rising, especially during the COVID-19 pandemic. The accumulating data supporting adverse effects of paternal substance use on male reproductive health and short-term and long-term offspring health outcomes are concerning. These findings are important as they support the notion that paternal — not only maternal — preconception exposure history can contribute substantially to successful and healthy conception, as well as to future offspring health. Comprehensive counselling by health-care providers, improved patient education and improved public health measures (especially targeting individuals interested in conceiving) focused on how paternal substance use can affect not only a patient's health but also the health of their future offspring are all needed.

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