Investigating the effects of nicotine on the male reproductive system

by
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Declaration

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Abstract

Much has been documented about the detrimental effects of adverse lifestyle factor exposure on the body. Exposure to factors, such as cigarette smoke, have proved to not only be a burden on global health and economy, but have also led to growing concerns about effects on systemic functions such as reproduction. The aim of the present study was to determine the effects of *in utero* and *in vitro* nicotine exposure on spermatozoal function and the antioxidant enzyme activity and lipid peroxidation (LPO) status of the male reproductive system. A better understanding of this process is necessary to combat the respective burdens of smoking and male infertility and for the prospective development of treatment strategies.

Two experimental models were employed: Wistar rats were exposed to nicotine *in utero* while human and rat spermatozoa were exposed to nicotine *in vitro*. *In utero* studies were achieved by selecting healthy pregnant rats and treating them with 1 mg/kg-bodyweight/day nicotine or 1 ml/kg-bodyweight/day 0.85% physiologic saline throughout gestation and lactation. Male rat pups were selected and sacrificed at each of the following age groups (n=6): 42 days, 84 days and 168 days old. The pups were only exposed to the treatment/saline via placental uptake or lactation. Biochemical analyses of the tissue comprised of measurement of LPO and antioxidant enzyme activity. Results indicated a significant association of maternal nicotine exposure to decreased levels of primary antioxidant enzymes in rat testes. Of particular note was the observation that the treatment group, of which each of the respective antioxidant enzyme levels were significantly less than the control group, was the oldest (d168) rat group.

In vitro studies were achieved by collecting sperm samples from healthy human donors (n=12), healthy rats (n=6) and obese rats (n=6). Samples were washed and exposed to different concentrations of high levels of nicotine (Control, 0.1mM, 1mM, 5mM, 10mM) *in vitro*. Semen parameters such as motility, viability and acrosome reaction were monitored at different time points (30min, 60min, 120min, 180min). Results revealed increasing *in vitro*

nicotine concentrations were associated with decreased viability and acrosomal status of human spermatozoa and decreased progressive motility and viability of rat spermatozoa. Obesity was also associated with decreases in progressive motility and viability of rat spermatozoa.

These results indicate that the acute *in vitro* exposure of spermatozoa to high levels of nicotine could adversely affect semen quality and may be an additive factor to the impediment of male fertility. *In utero* results reveal maternal nicotine exposure adversely affects male fertility in later life and seems to elicit more detrimental effects on the reproductive system than that of direct nicotine exposure to spermatozoa. Obesity also inhibits parameters of male fertility and these effects are exacerbated by nicotine exposure. The authors believe these adverse effects on the reproductive system to be related to an increased activation of leukocytes, excess production in reactive oxygen species (ROS) and consequent onset of oxidative stress (OS). Nevertheless this study agrees with other studies that nicotine exposure may be an additive factor to the impediment of male fertility.

Opsomming

Daar is reeds baie bekend oor die moontlik newe effekte vir die liggaam wat met 'n ongesonde lewenstyl gepaard gaan. Menslike blootstelling aan sulke faktore, soos sigaret rook, is wêreldwyd 'n las vir gesondheid en ekonomie en het gelei tot geweldige kommer onder navorsers oor die moontlike komplikasies vir liggaamlike funksies soos voortplanting. Die doel van die betrokke projek was om die effekte van *in utero* en *in vivo* nikotien blootstelling op die antioksiderende ensiem aktiwiteit en lipied peroksidasie status van reproduktiewe weefsel en die funksionele parameters van spermatozoa te bepaal. 'n Beter begrip van hierdie proses is noodsaaklik om die las van rook en vetsug teen te werk en vir die moontlike ontwikkeling van behandelingsstrategieë.

Twee eksperimentele modelle is ontwerp: Wistar rotte is *in utero* blootgestel aan nikotien terwyl mens- en rot- spermatosoë ook *in vitro* aan nikotien blootgestel is. Vir die *in utero* studie is gesonde dragtige rotte gedurende swangerskap en laktasie met 1 mg/kg-liggaamsgewig/dag nikotien of 1 ml/kg-liggaamsgewig/dag 0.85% fisiologiese soutoplossing behandel. Manlike welpies is gekies en geoffer op elk van die volgende ouderdomme (n=6): 42 dae, 84 dae en 168 dae. Die welpies is slegs aan nikotien blootgestel deur plasentale opname en laktasie. Biochemiese analise van die testikulêre weefsel het 'n beduidende assosiasie getoon tussen maternale nikotien blootstelling en verminderde vlakke van die primêre antioksiderende ensieme. Die 168 dag oue groep het 'n merkbare vermindering getoon tussen kontrole en nikotien weefsel vir elk van die antioksiderende ensieme.

Vir die *in vitro* studie is sperm monsters verkry vanaf gesonde mans (n=12), gesonde rotte (n=6) en vet rotte (n=6). Monsters is gewas en *in vitro* blootgestel aan verskeie hoë vlakke van nikotien (kontrole, 0.1mM, 1mM, 5mM, 10mM). Seminale parameters soos motiliteit, lewensvatbaarheid en akrosoom status is by verskei tydpunte gemeet (30min, 60min, 120min, 180min). Dit blyk dat verhoging in *in vitro* nikotien konsentrasies verband hou met verlaagde lewensvatbaarheid en akrosoom status van menslike spermatosoë en verlaagde progressiewe motilteit en lewensvatbaarheid van rot spermatosoë. Vetsug is ook

geassosieer met verlagings in progressiewe beweeglikheid en lewensvatbaarheid van rot spermatosoë.

In utero resultate openbaar dat maternale nikotien blootstelling manlike vrugbaarheid nadelig beïnvloed in latere lewe en blyk dat dit meer van 'n nadelige uitwerking op die voortplantingstelsel het as dié van direkte nikotien blootstelling aan spermatosoë. *In vitro* blootstelling van spermatosoë aan hoë vlakke van nikotien, het wel ook semen kwaliteit nadelig beïnvloed. Vetsug inhibeer ook manlike vrugbaarheids parameters en hierdie effek word vererger deur nikotien blootstelling.

Die outeure glo dat hierdie nadelige uitwerking op die voortplantingstelsel verband hou met 'n verhoogde aktivering van leukosiete, oortollige produksie van reaktiewe suurstof spesies en die gevolglike aanvang van oksidatiewe stres bevorder. Hierdie studie stem wel ooreen met ander studies wat nikotien blootstelling bestempel as 'n bydraende faktor tot die struikelblok van manlike onvrugbaarheid.

This dissertation is dedicated to my parents:

Pieter and Jeanne Maartens

for their unconditional love, support and motivation.

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List of Abbreviations

Reactive Oxygen Species	(ROS)
Oxidative Stress	(OS)
Unexplained male infertility	(UMI)
Lipid Peroxidation	(LPO)
Hypothalamic-Pituitary Gland	(HPG)
Gonadotropin Releasing Hormone	(GnRH)
Follicle Stimulating Hormone	(FSH)
Luteinizing Hormone	(LH)
In Vitro Fertilization	(IVF)
Intracytoplasmic Sperm Injection	(ICSI)
Polycyclic Aromatic Hydrocarbons	(PAHs)
Benzo[a]pyrene	(B[a]P)
Superoxide Dismutase	(SOD)
Catalase	(CAT)
Glutathione	(GSH)
Reactive Nitrogen Species	(RNS)
ROS correlated with total antioxidant capacity	(ROS-TAC)
Body Mass Index	(BMI)
World Health Organization	(WHO)
Assisted Reproductive Technologies	(ART)

Institutional Review Board	(IRB)
Stellenbosch University Reproductive Research	(SURGG)
Bovine Serum Albumin	(BSA)
Computer Aided Sperm Analysis	(CASA)
Sperm Class Analyser	(SCA)
Curvilinear Velocity	(VCL)
Straight Line Velocity	(VSL)
Average Path Velocity	(VAP)
Amplitude of Lateral Head Displacement	(ALH)
Beat Cross-Frequency	(BCF)
Fluorescein Isothiocyanate-Labelled Pisum Sativum Agglutinin	(FITC-PSA)
Bicinchoninic Acid	(BCA)
Glutathione S-transferase	(GST)
Malondialdehyde	(MDA)
Thiobarbituric Acid Reactive Substance	(TBARS)
Standard Error Mean	(SEM)

Chapter 1: Introduction and Aim of Study

1.1.   Introduction

Seminal quality has deteriorated rapidly over the last 50 years, making it an increasingly prevalent and relevant issue in unexplained male infertility (UMI). Researchers believe that the ever-changing environmental and lifestyle conditions to which the human body is exposed throughout an entire lifespan contribute greatly to this decline in quality. Industrialization and technological advances give rise to a range of different factors such as exposure to chemicals and toxins, harmful environmental agents and adverse lifestyle factors, all of which the body and consequently the reproductive system has to cope with.[1-5]

Due to the vast influence of agriculture on the infrastructure of developing countries, such as South Africa, there are many people living in rural farming communities and other areas of low income such as urban outskirts. Isolation from proper education, housing and social services leave many working class individuals and their families living in poverty. Subsequently many of these community members, especially the youth of these communities, fall victim to substance abuse and adverse lifestyle choices such as smoking.[6-8]

Lifestyle factor influences during any phase (pre-natal, adolescence and adulthood) of human development can mediate mechanisms disturbing the morphologic-, endocrine-, antioxidant- or fertilizing -capacity of the reproductive system and can have severe and irreversible effects on spermatogenesis in an organism or its offspring.[9-15]

Previous studies have reported that exposure to nicotine decreases spermatozoal functional parameters such as motility, count and normal morphology.[16-20] Many other studies have, however, disputed such findings arguing that nicotine does not affect spermatozoal function and that adverse reproductive parameters amongst smokers is attributable to other or the combined effect of other smoke constituents.[21-24] Results reported from research on the

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effect of nicotine on reproductive tissue have thus been clouded by methodological concerns and conflicting results and thus there have been few studies that have successfully assessed at what physiological concentration and to what extent nicotine affects the male reproductive system. Nonetheless, there is wide consensus amongst researchers that cigarette smoke, containing a multitude of chemical components, is detrimental to an individual's health. This may have negative implications for reproduction and should continue to be the subject of intensive research in the hope of, at least partially, alleviating some of the societal burden.

1.2.   Aim and Objectives of Study

The aim of the study was to determine the effect of *in utero* and *in vitro* nicotine exposure on the antioxidant- profile and the spermatozoal function of the male reproductive system.

The objectives of the present study were to evaluate:

- The effect of *in utero* nicotine exposure on the antioxidant enzyme activity and lipid peroxidation (LPO) of the testicular tissue in male Wistar rats of different ages (Model 1).
- The effects of high levels of *in vitro* nicotine exposure on the functional parameters of human spermatozoa [Model 2(a)].
- The effects of high levels of *in vitro* nicotine exposure on the functional parameters of spermatozoa from Wistar rats [Model (2)b].
- The effects of high levels of *in vitro* nicotine exposure on the functional parameters of spermatozoa from obese Wistar rats [Model 2(b)].

1.3.   Thesis Outlay

This thesis addresses one of the most prominent lifestyle factors currently affecting male infertility: cigarette smoke. Chapter 2 provides a brief overview of cigarette smoke and the possible effects that may occur due to exposure to the male reproductive system. Materials

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and methods of the ensuing study follow in chapter 3, while chapter 4 and 5 report the results and discussion of the findings respectively.

1.4.   Conclusion

The literature certainly provides enough compelling evidence to conclude that adverse lifestyle choices such as smoking play at least some role, if not a definitive one, in the development of UMI. This process can be monitored by examining tissue and spermatozoa exposed to nicotine subjecting them to biochemical analysis and semen analysis. By treating tissue or cells of both human and rat subjects and by exposing them to nicotine prenatally and directly this study will provide a unique, comparable and valuable visual of the effect of nicotine on the male reproductive system. By exposing spermatozoa to nicotine *in vitro* the study will contribute to the understanding of direct nicotine exposure to cells and cast light on the difference and relevance between *in vitro* studies and *in vivo* studies in the field of nicotine exposure and reproductive biology. The results obtained from this study will contribute to the understanding of the effect of nicotine on the body and will be beneficial to development of treatment strategies and will help combat the respective burdens of smoking and male infertility.

Chapter 2: Literature Review

2.1.   Introduction

Infertility: Reduced or absent (possibly reversible) capacity of a man and/or a woman to reproduce.[25] Infertility is generally used to reference the reproductive state of a couple who are sexually active without the use of contraceptives and yet are unable to achieve spontaneous natural pregnancy after a year of attempt. Infertility stems from both male and female reproductive impediments. Though the range of diagnostic tools, tests and treatments have developed at an exponential rate in recent times; it is estimated that 5% of couples, nearly half the number of people seeking fertility treatment, that remain unwillingly infertile. *Unexplained infertility:* is the term used to describe the reproductive state of a couple who are infertile despite displaying normal female fertility parameters as well as male seminal parameters within the expected ranges for successful reproduction. Prevalence is estimated at between 6% and 27%, subject to the comprehensiveness of diagnostic effort.[1-4] The inability of modern medicine to explain the phenomenon of unexplained infertility has attracted the interest of many researchers worldwide and several possible causes have been investigated including morphologic, molecular and genetic defects (male and female), coital difficulties such as erectile dysfunction, autoimmune infertility and spermatozoal dysfunction.[5] However, few of the theories have had concrete results such as successful treatment strategies. Successful reproduction, it seems, remains ever elusive to many hopeful couples presenting with unexplained infertility.

Seminal quality has deteriorated rapidly over the last 50 years, making it an increasingly prevalent and relevant issue to UMI. Researchers believe that the ever-changing environmental and lifestyle conditions to which the human body is exposed throughout an average lifetime, contribute greatly to this deterioration. Developments in industry and changes in technology give rise to a range of different factors such as exposure to chemicals and toxins, harmful environmental agents and adverse lifestyle factors, all of which the body

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and consequently the reproductive system have to cope with. Environmental insults during the maternal and infancy phases of human development can mediate mechanisms disturbing the morphologic-, endocrine-, fertilization- or antioxidant- aspects of testicular tissue and can have severe and irreversible effects on spermatogenesis (spermatozoal production in the mature testes) in a subject or its offspring. This can furthermore adversely affect seminal parameters.

2.2. Male Development and Infertility

Male reproductive development starts with gender determination: when the Y-chromosome of a spermatozoa fuses with the X-chromosome of an oocyte creating an XY coded zygote. Thereafter, gonadal differentiation initiates as the foundation for testicular development. The bipotential gonads differentiate from the genital ridge which forms as a thickening of somatic cells on the surface of the mesonephros. After the gonadal differentiation process is completed, sexual differentiation starts. The gonad gives rise to three bipotential cell lineages that differentiate into steroidogenic cells, Sertoli cells and cells responsible for completion of gonadal structural development. The first foetal precursor cells are responsible for the formation of steroidogenic cells that are responsible for the secretion of sex hormones and subsequent onset of secondary sexual characteristics. The second cell lineage gives origin to Sertoli- and mesenchymal cells. The Sertoli cells regulate the synthesis of the seminiferous tubules, while the mesenchymal cells differentiate into Leydig cells. The third cell lineage differentiates into the gonad structure. Development of the bipotential gonad is dependent on the anti-Mullerian hormone secreted by the Sertoli cells, with testosterone being secreted by interstitial cells and the insulin-like 3 hormone. The intermediate mesoderm is homologous for both male and female development and gives rise to the Wolffian ducts, Mullerian ducts and the gonad precursors. During male development the Mullerian duct dissolves away while the Wolffian duct gives rise to the epididymis, vas deferens, ductus deferens, ejaculatory duct and the seminal vesicle. The development of the external male genitalia is dependent on dihydrotestosterone exposed to the foetus during

the third trimester of pregnancy. The transfer of the testes from the genital ridge to the scrotum is a process of cardinal importance to sexual differentiation. Testosterone induces the relaxation of the cranial suspensory ligaments allowing the descent of the testes into the scrotum. The increased abdominal pressure due to the viscera growth and the elastic properties of the testes then cause the testes to be forced through the inguinal canal and into the scrotum. After the initial development of the essential male reproductive organs, the reproductive system lies dormant until puberty when the Hypothalamic-Pituitary Gonadal (HPG) axis becomes active and the process of spermatogenesis is initiated. The precursor cell development is of cardinal importance to spermatogenesis in the adult male. It is crucial that precursor cells proliferate unimpeded and give rise to an optimal amount of spermatogonia in later life.[5,26-27]

With the onset of puberty, the hypothalamus secretes Gonadotropin Releasing Hormone (GnRH), which stimulates the anterior pituitary causing the secretion of the gonadotropins namely: Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH). LH is responsible for the stimulation of Leydig cells to produce testosterone and thus promote the onset of secondary sexual characteristics. FSH is responsible for the stimulation of Sertoli cells responsible for the onset of spermatogenesis.[26-29]

Spermatogenesis: is the process whereby spermatozoa are produced.[25] In the mature male reproductive system, spermatogenesis is responsible for the production of haploid gametes from diploid spermatogonia during a complex and delicate process that initiates during puberty and continues throughout the male individual's lifetime. Spermatogenesis requires a combination of synchronized gene expression and cell division and takes place in the testes over a period of just more than two months.

Of fundamental importance to the normal occurrence of spermatogenesis are the Sertoli cells as they alter rates of spermatozoal production in adult testes and produce factors essential to gamete development.[30-32] Leydig cells are responsible for the secretion of

androgenic hormones. These androgens are key to appropriate testicular development, such as urethral groove fusion and descent of the testis.[33-34]

Due to the intricacy of the process, spermatogenesis is totally dependent on the existence of optimal conditions. It is extremely sensitive to changes in the external environment. Therefore environmental and lifestyle insults that affect gonadal differentiation, Sertoli- or Leydig- cell proliferation or spermatogenesis at any age could affect male reproductive development and thus lead to adverse reproductive pathologies such as oligozoospermia, asthenozoospermia, hypospadias, testicular spermatogonia cancer and cryptorchidism. [27-30]

2.3.   The Environment and Male Infertility

The male reproductive system can be exposed to adverse environmental factors during any of its three stages of development i.e. maternal-dependent (gestation and lactation) development, early-life (pre-pubertal) development or adulthood (sexual maturity). The effects of environmental factors can be extremely detrimental, specifically during the two developmental stages (maternal/early-life), to testicular development and spermatogenesis. This can result in poor semen parameters later in life, including impaired spermatozoal concentration and motility. Direct exposure to unfavourable environmental factors can also occur during adulthood. Similar to the maternal and infancy stages of development, such factors can have a negative impact on spermatogenesis. However, direct exposure during later life is regarded as reversible while early life exposure is considered to be irreversible.[35-36] Exposure to adverse elements can impair the male reproductive system during any of its stages of maturity and thus affect spermatogenesis through several mechanisms of action as summarized in Table 2.1 below:

Table 2.1: Environmental insults during male development.

Stage of Development	Target	Effect	Example of insult
Maternal Exposure	Reproductive Organ Development	Decreased Spermatogenesis	Lifestyle, Endocrine Inhibiting Substances
Pre-Pubertal	Scrotal Tissue	Decreased Spermatogenesis	Fluctuations in Temperature
Post-Pubertal	Sertoli/Leydig cell function	Decreased Spermatogenesis	Xenobiotics, Lifestyle

During pregnancy, maternal lifestyle factors such as cigarette smoking can impact on the developing male foetus, resulting in reduced Sertoli cell count and spermatozoal concentration later in life. The components in cigarette smoke, for example, antagonize androgen receptor mediated function and thus impede reproductive organ development. Maternal obesity has also been shown to reduce spermatozoal concentration in the male offspring and inhibit testicular development via interference with the testosterone: oestrogen balance of the foetus. Maternal diet can also affect the developing foetus through the ingestion of anabolic steroids found in meat. These anabolic steroids and the oestrogenic substances used to process and cook the meat can act as xenobiotics, which are endocrine inhibiting, and impair the critical hormone balance in foetal development leading to impaired spermatogenesis in the mature offspring. Other harmful substances such as herbicides and pesticides, which are lipophilic, can also be absorbed and start amassing in the fat of pregnant mothers. These substances are then slowly released to the foetus and infant via placental uptake and breast-feeding.[9-15]

Fluctuations in postnatal thermal scrotal temperatures may lead to an adverse reproductive state known as scrotal heat stress. This can be responsible for a decline in spermatozoal count in later years. Studies have shown that the use of disposable plastic-lined diapers during infancy and early childhood, instead of reusable cotton diapers, may result in higher scrotal skin temperatures, impairing the incredibly sensitive temperature homeostasis of the developing testis.[37-39]

Environmental insults in the form of oestrogens have been shown to cause responsive changes in the neuroendocrine system of the mature male with effects notable in reproductive function and spermatogenesis.[40] Such environmental oestrogens, known as xenobiotics, can have a negative impact on male fertility as ingestion of these substances has been directly correlated to decreased spermatozoal concentration.

Xenobiotics have been found to adversely affect the male reproductive system in the following ways:

- The inhibition of FSH secretion by the foetal pituitary gland and thus a disturbance in the HPG-axis leading to a decreased number of Sertoli cells.

- The inhibition of Leydig cell formation and function leading to decreased testosterone production and decreased gamete differentiation.

- The inhibition of androgen receptors within the foetal testes.

- The conversion of xenobiotics to quinones that produce Reactive Oxygen Species (ROS) that, when produced in excess, induce oxidative stress (OS) or damage DNA.[41]

2.4.　　Lifestyle Factors, Smoking and Prevalence

As already noted, it is increasingly evident that spermatogenesis is an immensely sensitive and delicate process which is dependent on optimal conditions and is severely susceptible to fluctuations in external factors.[27] The rapid expansion in the modern Western lifestyle with its concomitant increase in industry and associated chemicals as well as changes in diet, exercise, alcohol intake, smoking habits and stress is probably at least, if not solely, responsible for the notable decrease in male fertility.

Cigarette smoking: is the act of burning and inhaling tobacco (originating from the *Nicotiana tabacum* plant) generating amongst others nicotine, an addictive, sympathomimetic, thermo genic reagent which alters metabolism by for e.g. increasing resting energy usage. The smoke also contains many carcinogenic substances which result in adverse health

consequences such as the advancement of atherosclerosis, cancer, emphysema, cardiovascular disease and stroke.[25]

In recent years, much has been documented on the detrimental effects of cigarette smoking and associated nicotine exposure on the body. Despite knowledge of the harmful effects of nicotine and high government taxation, the WHO estimates smoking to be associated with approximately one third of the world's population older than 15 years of age greatly increasing incidence of diabetes, cancer, emphysema, cardiovascular disease and stroke.[21,42-45] Due to the vast influence of agriculture on the infrastructure, many farming communities are found in South Africa. The rural setting and its isolation from proper education, housing and social services, leave many of the working class individuals and their families living in poverty. Subsequently, many of these community members, especially the youth of these communities, fall victim to substance abuse. This unsettling set of circumstances in addition to the already problematic number of people addicted to drugs and alcohol in the ever-growing urban areas of South Africa, has led to the acknowledgement of substance abuse as an enormous problem affecting the country. Research has shown that the five substances most easily obtainable and responsible for these exceptionally high figures are as follows: cigarettes, methamphetamine, mathaqualone, alcohol and cannabis.[46-48]

Current research estimates that 20-34% of South Africans smoke.[6-7,43] A recent prevalence study conducted in Cape Town and the surrounding metropolitan revealed 27% of high school children partake in nicotine usage in the form of tobacco smoke.[8] A study amongst students undergoing tertiary education yielded that 15% of male students and 5% of female students regularly use cigarettes or an equivalent tobacco product.[49] The effects of this adverse lifestyle choice are clearly visible in the country's mortality rates. Prevalence studies showed that 4525 people died due to lung cancer in 2006, a known complication of long-term exposure to cigarette smoke, and in the year 2000, between 41000 and 46000 deaths were attributable to smoking.[42,50-51] These statistics are not as morbid as when compared to

countries such as the United States of America where smoking related deaths approximate 440000 per year (population of 313.9 million)[52] or the United Kingdom which has an annual lung cancer death toll of 34000 deaths (population of 62.74 million)[53], however when taken into account the already dire economic situations that third world countries face and that smoking is becoming an ever prevalent phenomenon in such countries- the need for preventative measures and further research is undeniable. This burden to South Africa and other comparable third world countries health and economy has led to great concern amongst researchers about the effects of cigarette smoke on the body's systemic functions.

2.4.1. Smoking and Addiction

Smoking exposes the body to more than 4000 chemicals that have the potential to harm, such as nicotine, carbon monoxide, nitrosamines, alkaloids and hydroxycotinine. Of these substances nicotine has been identified as one of the key substances responsible for addiction to cigarette smoke and adverse health implications. Nicotine is craved for its ability to stimulate the body (similar sensation to caffeine) and then relax the body leaving the consumer with a sense of minor euphoria. A cigarette contains on average between 8-20 mg of nicotine, yet only about 1 mg is absorbed by the body. Nicotine can be absorbed into the blood stream through the skin, the lungs (primary site of uptake) and/or the mucous membranes. After absorption nicotine is transported via the blood, to the brain and to the other tissues in the body.[54] The brain and body then undergo a characteristic biphasic transition of stimulation/relaxation. The stimulatory effect is caused by stimulation of the sympathetic systems and subsequent adrenaline secretion causing elevated heart rate, respiration and the release of energy from bodily stores. The relaxation effect is caused by the ability of nicotine to act as an agonist of the neurotransmitter acetylcholine and its receptors and thus affect metabolism, efferent signalling and basic brain function such as learning and memory. The increased secretion of acetylcholine stimulates cholinergic pathways and the so-called reward pathways of the brain, dopamine and other endorphins in particular, which are responsible for the above mentioned euphoric sensation. Acetylcholine

secretion also stimulates release of glutamate, involved in learning and memory, causing a memory loop of the positive sensation and reinforcing the nicotine craving.[54-58] Nicotine has shown possible therapeutic potentials in neurodegenerative diseases such as Alzheimer's disease and Tourette's syndrome but nicotine exposure has proved to have definite adverse pathophysiologic complications such as cancer, emphysema, cardiovascular disease and stroke.[21,42-43]

2.4.2. Smoking and Infertility

Smokers and their offspring are subjected to a bombardment of tobacco combustion products causing an enormous developmental risk of genetic and other reproductive defects. The consequences may not be apparent immediately at birth but can manifest later in life. It is therefore imperative that anti-smoking measures be implemented, that prospective parents be strongly encouraged to give up smoking and supported in their efforts to do so.

2.4.2.1. Effects of Smoking on Male Fertility

Spermatozoal Parameters and Seminal quality

Chronic smoke exposure has been positively correlated to lower spermatozoal concentration, decreased motility and increased abnormal morphology. [56-57,59-62] Spermatozoal motility has also shown a negative correlation to the amount of cotinine and hydroxycotinine in the seminal plasma. Asthenozoospermia, or reduced spermatozoal motility, may be an early indicator of reduced semen quality in light smokers. The incidence of teratozoospermia, or increased abnormal spermatozoal morphology, was also significantly higher in heavy smokers than in non-smokers.[57,63-66]

Maternal exposure to cigarette smoke has long-term consequences on the fertility of male offspring. Male offspring exposed to cigarette smoke *in-utero* have proved to have lower spermatozoal counts, decreased fecundity and reduced numbers of morphologically normal spermatozoa. These adverse effects seem to become evident during adolescence.[9-10,16,67] *In utero* paternal exposure to cigarette smoke has also been correlated to reduction in the male

to female ratio of offspring (a reduction in frequency of male births). Study results seem to indicate that this reduction in sex ratio originates around time of conception and investigators suspect the involvement of periconceptional paternal exposure to toxic environmental factors such as paternal smoking.[68]

Fertilization Capacity

Several studies concluded that smoking adversely affects the fertilization capacity of spermatozoa, embryo cleavage rates and decreased fecundability.[63-65,69-70] These findings are in accordance with studies reporting the association of smoking and passive smoking of men and women with delayed conception.[62] In addition, studies have shown that smoking adversely affects outcomes of assisted reproductive techniques such as *In Vitro* Fertilization (IVF) and Intracytoplasmic Sperm Injection (ICSI). Researchers believe that this observation results from smoking-related altered DNA resulting in compromised fertilization and/or compromised embryo development.[66]

2.4.2.2. Possible Causes of Fertility Impairment

Nicotine and Other Smoking Components and Metabolites

Several substances originating from cigarette smoke have been identified and are being intensely researched for their possible effects on fertility. Cigarettes contain and release several carcinogens and mutagens into the bloodstream.[62] The primary metabolites found in the body after smoking are nicotine and cotinine. Nicotine is the substance responsible for addiction to smoking and exposure can result from any or multiple of the following sources:

- Usage of any tobacco-related product such as cigarettes, cigars, snuff and chewing tobacco.
- So called 'second hand smoking' or passive smoking- exposure to somebody else's smoke in close vicinity.
- Occupational exposure during tobacco extraction.
- Environmental exposure as nicotine has been used as an insecticide in the past.

- Nicotine patches.

- Maternal smoking can cause nicotine exposure via placental crossing *in utero* or through postnatal breast milk ingestion.[52,54]

Nicotine has a half-life of about 2 hours in the body, during which time 70-80% is metabolized to cotinine by cytochrome P450 and the rest is excreted in the urine, faeces, etc. Cotinine is the major metabolite of nicotine and has a half-life of about 20 hours in the body.[71-72] Nicotine and cotinine have been found in serum, urine, saliva and milk and in smokers' seminal plasma in subjects exposed to environmental tobacco smoke. Nicotine concentrations of between 70µg/l (0.00043mM) and 300µg/l (0.00185mM) are commonly found in the semen of casual (1-10 cigarettes/day) and habitual smokers (>30 cigarettes/day).[22-24,73-74] The effects of nicotine and cotinine are tabulated below (Table 2.2) along with two of the major combustion agents associated with cigarette smoke.

Table 2.2: Major components of cigarette smoke and their systemic effects.

Substance	Origin	Target	Effect	Reference
Nicotine	Metabolite	Spermatozoal Function, Androgen Function, Obstetrical Outcome	decreased motility, sperm count, increased abnormal sperm, decreased male libido, reduced litter weight and size	9-11,35-36,79
Cotinine	Metabolite	Spermatozoal Function	abnormal sperm morphology, decreased motility, decreased capacitation status, altered membrane function	80,134
Polycyclic Aromatic Hydrocarbons (PAH's)	Combustion Agent	Hydrocarbon Receptors	endocrine disruption, anti-androgenic effects	30,134
Benzo[a]pyrene (B[a]P)	Combustion Agent	Spermatozoal DNA	DNA adducts leading to DNA-mutations	30,134

Although several components of cigarettes have been researched for their possible adverse effects on reproduction, study results have not been produced without methodological criticisms.[21,22-24] The general consensus amongst researchers is, however, that cigarette

smoke with all its components combined is detrimental to an individual's health and has negative implications for reproduction and should be strongly discouraged.[75-78]

Developmental Deficiencies

Cigarette smoking during pregnancy reduces Sertoli cell count. Researchers attribute this observation to components in cigarette smoke antagonizing androgen receptor mediated function and thus impeding reproductive organ development.[9-11] This hypothesis is concurrent with studies that concluded that nicotine and cotinine found in smoke either inhibit the intracellular calcium content or completely block the effects of calcium on steroidogenesis in Leydig cells, resulting in a decline in circulating testosterone levels. Secretory dysfunctions in Leydig cells could also be the cause of observed smoking-related deficiency in spermatozoal maturation and spermatogenesis.[67,69,79-80] Cellular dysfunction associated with smoking could also affect circulating FSH levels. Smoking reduced FSH secretion by 17% in smokers and people who smoke >10 cigarettes per day presented with 37% less FSH levels than non-smokers.[67] These findings are in agreement with studies reporting that pre- and perinatal administration of nicotine, as a component of cigarette smoke, decreased circulating testosterone levels and affected subsequent secondary sexual characteristics.[81]

In utero exposure to smoke has been associated with decreased testes size. This is believed to be a result of smoking impediment to foetal gonadal development.[82] Smoking has also been associated with the retention of cytoplasm by spermatozoa. Spermatozoa normally secrete excess cytoplasm before they are released by the germinal epithelium or during transit through- or maturation in- the epididymis. Retention of cytoplasm by spermatozoa is a characteristic associated with infertility.[83]

Reactive Oxygen Species and Oxidative Stress

Free radicals are highly reactive compounds, attributed to an uneven number of electrons in their atomic structure causing them to easily react with other substances. Free radicals such

as ROS are natural products of the body's aerobic metabolic processes. Free radicals have short-term functions in the body such as acting as second messengers in signaling cascades or for use in immune function. When utilized in normal bodily processes any damage caused by free radicals is easily dealt with by cell detoxification. However, when these free radicals are produced in excess they can have deleterious effects on cells and tissue by attacking and damaging proteins, lipids and DNA in the cell. Superoxide for example, is converted to a much more aggressive species during OS and is capable of much damage by way of ATP depletion. This imbalance in the body is termed OS and refers to a state of imbalance in the production of free radicals in the body and the body's ability to detoxify these substances or deal with the damage they cause. Long-term OS causes cells to become necrotic and is associated with many adverse effects and diseased states in the body.[84-85,143]

Cells and tissue have to maintain a delicate homeostasis between the production of free radicals, the elimination of free radicals and repair of the damage they cause. Cells have defence mechanisms that solely see to this purpose by inactivating excess unstable compounds by process of immediate elimination. These defence mechanisms mainly consist of antioxidant and other redox molecules that neutralize free radicals at cellular level. Antioxidant enzymes neutralize the free radicals by either partly absorbing some of the oxidant's energy (in the form of electrons) or giving up electrons to bind and stabilize the radical. Antioxidants also inhibit the production of free radicals and limit the damage they cause. The human body produces several antioxidant enzymes of which the most well documented are the primary antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH). These enzymes maintain the homeostasis between free radical production and inactivation by converting free radicals as follows (Figure 2.1).[86]

Superoxide Dismutase:

•   Neutralizes superoxide anions by forming hydrogen peroxide and oxygen as products. $\longrightarrow$

        $2O_2^- + 2H^+$ $H_2O_2 + O_2$

Catalase and Glutathione: $\longrightarrow$
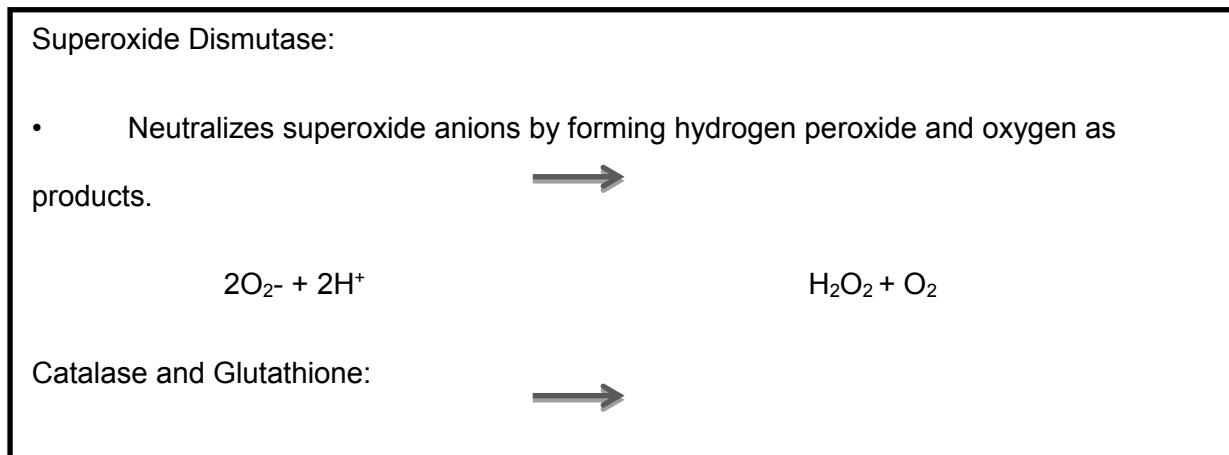
Figure 2.1: The primary antioxidant enzymes and their function.

Cigarette smoking results in the release of several combustion-, mutagenic- and carcinogenic -agents into the circulation. These substances can increase the production of free radicals such as ROS and Reactive Nitrogen Species (RNS) which, when produced at pathophysiologic levels, can lead to OS and ultimately to infertility.[56-57,60-61,87-89]Furthermore, smoking exposure can cause DNA fragmentation and even cause β-cell apoptosis exacerbating OS.[88,90-91] Cadmium is a heavy metal known for its detrimental effects to reproduction. Studies have reported that semen of smokers present a 100-fold increase in OS and up to 5x cadmium levels. Smoking was also correlated with a higher incidence of teratozoospermia.[60,92]

Cigarette smoke has a direct correlation to decreased levels of seminal plasma antioxidants including Vitamin C and Vitamin E.[93-94] Studies support this hypothesis by correlating an increase in ROS levels with decreased total antioxidant capacity (ROS-TAC) scores.[60] ROS also targets polyunsaturated fatty acids in cell membranes, decomposing them and causing impairment of membrane fluidity as well as the production of several oxidants which are toxic to cells. This process is known as lipid peroxidation (LPO). Studies have also observed a decrease in antioxidant enzyme protection in the reproductive system due to smoking. Smoking significantly reduced GSH, SOD and CAT concentrations. These antioxidant

enzymes are key to cell protection which, when inhibited, may significantly reduce fertilization rates.[94-97]

Researchers believe that the generation of ROS and subsequent development of OS is related to leukocyte activation. It is understood that metabolites that enter the circulation as a result of smoking (such as PAH's) act as chemotactic stimuli inducing inflammation and attracting leukocytes into the seminal plasma which produce ROS and induce OS. Smoking has been proved to increase incidence of leukocytospermia or excessive leukocytes in the seminal fluid. Production of ROS may also impair the mitochondrial genome, impair the electron transport chain and thus exacerbate OS.[60,67]

Testes, Hypoxia and Osmolarity

The reproductive system has very specific requirements for optimal function. The testes are anatomically very sensitive to changes in oxygen supply and osmolarity. Smoking has proved to disrupt both the oxygen- and moisture- supplies to tissues [56-57,60-61] Nicotine intake results in increased circulating cholesterol levels promoting the onset of atherosclerosis in arteries supplying blood to the reproductive system and thus resulting in a lowered blood supply.[98-99] Chronic exposure of the male reproductive system to nicotine leads to a state of vasoconstriction or vasospasm (as part of the sympathetic nervous system activation) within the penile arteries and smooth muscle, leading to an impairment of Leydig cell function.[69,100] As previously stated smoking also, in a paradoxical fashion, stimulates the production of RNS (nitric oxide) which acts as a vasodilator and thus further impairs functional homeostasis of nutrient supply to testicular tissue.

Smoking has also been associated with decreased semen volume. Due to the fact that smoking increases metabolism and subsequent water utilization in the body, it is possible that smoking might affect the very sensitive osmolality state of the testes and thus disrupt testicular processes such as spermatogenesis resulting in subsequent impediment of spermatozoal parameters.[97]

18

Genetics

As previously mentioned, smoking has been reported to cause DNA fragmentation. Altered DNA has proved to increase apoptotic markers and promote the onset of OS. Altered DNA might also lead to DNA adduct formation and compromised -fertilization and –pregnancy outcomes and it could lead to decreased reproductive parameters in progeny and even the onset of childhood cancer. [11,78,88-90,101] Studies showed cigarette smoking to increase spermatozoal aneuploidy and disomic spermatozoa with increased risk for compromised fertility and for spermatozoal disomy in progeny.[102] Assisted conception studies showed that germinal cells are at risk to genetic damage from smoking, but can repair during meiosis. Spermatozoa do not, however, have a large capacity to repair. Smoking affects the meiotic spindle of spermatozoa leading to chromosome errors and adverse fertilization outcomes.[103] Researchers also believe that lifestyle factors, such as cigarette smoke, might alter genetic material and affect systemic functions of progeny. *Transgenerational Inheritance:* refers to the ability of environmental factors to not only promote a pathophysiologic condition in an individual, but to promote it in successive generations. Most environmental parameters have the ability to alter the epigenome. Mutations in the gametes and gamete production line, which become irreversible, can cause transmission of genetic phenotypes between generations and can cause downstream harm to the testes and subsequent seminal parameters of the progeny.[104-106]

Cadmium and Zinc

Studies have correlated smoking with an increase in cadmium levels. Cadmium has been found to be directly connected to male fertility problems. Cadmium levels are higher in the seminal plasma and blood of infertile men than that of fertile men. It affects the male reproductive system in several ways. Cadmium exposure is directly correlated to blood vessel toxicity. It inhibits spermatozoal concentration and motility due to its antisteroidogenic properties that lead to a lowered testosterone secretion. Studies have attributed the

antisteroidogenic observations associated with cadmium exposure to extreme Leydig-cell toxicity. Cadmium causes the following adverse effects in Leydig cells:

- Decreased cell viability.

- Decreased testosterone secretion.

- Increased malonaldehyde levels.

- Decreased SOD levels.

- Severe DNA damage.

Cadmium may disrupt inter-Sertoli cell tight junctions and thus disrupt the blood/testes barrier and consequently inhibit spermatogenesis. Cadmium exposure has been directly correlated to asthenozoospermia. It has also been found to have pro-oxidant properties that mediate generation of free radicals and reduce antioxidant levels such as zinc which are crucial to spermatogenesis.[107-110]

Smoking has also been linked to reduced levels of seminal zinc. Zinc is a key element to spermatogenesis and male reproduction and compromised seminal zinc levels could lead to adverse fertilization outcomes. Studies have reported that zinc supplements, when used in conjunction with folic acid, increased spermatozoal counts and even reversed the some of the detrimental effects of cadmium.[111-113]

2.4.3. Smoking and Co-dependence

Smoking has been reported to be synonymous with other substances that can adversely affect male fertility. Co-dependence studies are few in number, yet certain studies have shown a positive correlation between smoking and factors such as alcohol use and obesity.[114-117]

Obesity: is the abnormal accumulation of adipose tissue in the body due to a series of endogenous or exogenous factors. It is often synonymous with the development of adverse health consequences such as atherosclerosis, hypertension, hyperlipidaemia, insulin resistance and diabetes milletus.[25] Men with a body mass index (BMI) above 25 are at an up

to 3 times greater risk for being classified as infertile, due to reduced spermatozoal concentration and increased spermatozoal DNA fragmentation. There are several theories that attempt to explain the link between obesity and infertility:

- Studies have shown a direct correlation between change in BMI trends and changes in endocrine and exocrine functions of the testes. The prevalence of excess adipose tissue leads to the conversion of testosterone to oestrogen, increases in insulin and leptin and decreases in LH and FSH.

- Accumulation of inner thigh, pubic and abdominal fat could cause infertility through increased scrotal temperatures.

- Upper airway obstruction and subsequent hypoxia often results as a complication of obesity resulting in sleep apnoea. Thus nightly cycles of raised testosterone levels are interrupted leading to decreased circulating levels of testosterone and LH.

- Obesity is significantly associated with erectile dysfunction and decreased libido.

- Obesity is associated with induced states of systemic proinflammation. Systemic proinflammation is accompanied by increased activation of leukocytes, production of ROS and subsequent onset of OS and LPO which severely impairs spermatozoal parameters.[118-121]

Excessive chronic alcohol usage is detrimental to spermatogenesis and male fertility. Alcoholism has been associated with testicular atrophy, impotence, impaired libido, reduced FSH, LH and testosterone levels, OS, reduced antioxidants and LPO and severely impaired seminal parameters. Studies suggest that alcohol promotes the overproduction of free radicals such as ROS inducing a state of OS in the testes as well as inducing hypoxia and causing tissue damage in a system already very sensitive to changes in oxygen supply. Excessive alcohol intake also causes an increase in circulating levels of oestrogens in males disrupting the normal production of testosterone and reducing the secretion of LH adversely affecting the maturation of spermatozoa.[122-125]

2.5.   Looking Forward: Possible Treatment Solutions of Environmental Insults to the Reproductive System

The treatment of UMI does not benefit from the convention of standard protocol and clinical practice decision making. Such circumstances call for a specific scientific plan to identify and address a known defect and the subsequent applicable risk and treatment management.[1] There are two forms of UMI management that can be followed: expectant management and interventional management. Expectant management entails the regulation of environmental and lifestyle factors to such an extent as to better spermatozoal functional parameters and thus better chances for successful conception. Interventional management entails any form of assistance to conception whether invasive such as surgical or pharmacological via the use of oral medication.

Expectant management is to be recommended if the woman is less than or between 28–30 years of age and the duration of unsuccessful infertility is less than 2–3 years.[59] Lifestyle factors that are addressed and managed are chemicals, smoking, nutrition, exercise and environmental pollution.

The children of smokers are subject to a bombardment of tobacco combustion products, via *in utero* and passive smoking, causing an enormous developmental risk of genetic defects. The consequences may not be apparent immediately at birth but can manifest later in life. It is therefore imperative that anti-smoking measures continue to be implemented, that prospective parents be strongly encouraged to give up smoking and supported in their efforts to do so as cessation has proved to be the most effective treatment to smoking ailments.[126]

The amount of evidence on the known health benefits of regular exercise and balanced nutritious diets as well as the detrimental effect of obesity on the reproductive system is overwhelming. Fertility is decreased by being either overweight or underweight. More research into the effect of diet and exercise on fertility is needed. For time being, people

trying to conceive should be advised to exercise moderately and aim to have a BMI between 20 and 25 kg/m^2.

Interventional management which includes medication, surgery or assisted conception is justified in cases of unexplained infertility of long duration and/or advanced maternal and paternal age. In recent times, management has been solely based on surgical treatments or antioxidant-based treatments.

In general terms antioxidants are compounds which scavenge, suppress and dispose of ROS. The major antioxidants are vitamin A, vitamin E, beta-carotene, vitamin C and the trace mineral selenium. A number of nutritional therapies have been shown to improve spermatozoal counts and spermatozoal motility, including antioxidant enzyme-, phytonutrient-, carnitine-, arginine-, zinc- and vitamin B-12 -therapy. Folic acid and zinc supplements, when used in combination have been shown to increase spermatozoal counts in a placebo-controlled trial. Antioxidants like GSH, and coenzyme Q10, have also proven beneficial in treating male infertility.[112,127-128]

Surgical treatment can be an effective approach in the treatment of male infertility of defective morphologic origin such as obstructive azoospermia. Accurate identification of the cause of infertility and microsurgical approaches will often provide effective treatment with low morbidity rates. Appropriate training in microsurgery and overall experience with surgical techniques will produce the most effective treatment of the infertile man.

2.6.　　Shortcomings/Constraints of Current Research on UMI and Environmental Influences

The diagnosis, evaluation and treatment recommendations for male infertility are normally based on a standard semen analysis even though diagnostic tools, tests and parameter guidelines are limited and vary between urological societies, fertility clinics and the World Health Organization (WHO) laboratory manual for the processing of human semen editions.[129-131] The parameters for which guidelines are laid out (spermatozoal concentration,

motility and morphology) also vary between different ejaculates collected from the same individual. This variation in samples is attributed to sexual activity and abstinence, function of accessory reproductive glands and environmental exposures and thus cannot be regarded as accurate and finitely representative of an individual's fertility profile.

Analyses of the results of studies evaluating effects of environmental factors on the male reproductive system are limited in the sense that they are inconsistent in the use of biological analytical techniques, controlling for variance in factors and weakness of study design. *In vivo* studies done on animals may also prove to have limited applicability to human equivalents such as gestation and lactation. Developmental periods in animals are much shorter than in humans. Treatment with a factor and the cessation and measurement of its effect is much easier in the controlled environment under which such experiments are conducted on animals than in the complex, uncontrolled and chaotic conditions under which humans live. Animals, as opposed to humans, vary much less between subjects in terms of their spermatogenesis profile. Humans are exposed to a mixture of environmental chemicals, toxins and other factors so that isolating and interpreting the effect of a single factor without interference by other parameters is near impossible.[132-133]

Diagnostics such as comprehensive history investigation, and molecular and genetic inquiry, should be employed and should be complemented with an all-inclusive physical examination before conclusions are drawn and treatment plans are based on fertility status. The use of the most recent updated WHO reference values in clinical practice will likely change the classification of infertility for many couples. Rapidly developing technology and constant change in seminal parameter baselines emphasize the need for clinicians to consider a much more diverse variety of parameters than just spermatozoal concentration and motility when assessing male subfertility.

Assisted Reproductive Technologies (ART) might be an interim solution to the problem of UMI - but as a technique it is not without its difficulties and shortcomings and refinement through research is required.[1] ART has allowed a large proportion of men who are

experiencing fertility problems to procreate. As amazing as this technology is, it is a mere treatment of the symptom and not of the actual causal problem and thus is counterproductive, in a sense, to understanding the causes, effects and possible treatments of male infertility. It is of cardinal importance that research be done on UMI and the effects of environmental exposure on the reproductive system so that we may better our understanding of and hope for solutions to the prevention and cure of this growing and topical problem.

2.7.   Conclusion

Seminal quality has deteriorated rapidly in the last 50 years, making it an increasingly prevalent and relevant issue to UMI. Researchers believe that the ever changing environmental and lifestyle conditions to which the human body is exposed throughout an entire lifespan are of paramount importance in this occurrence. Modern day developments in industry and changes in lifestyle have led to a range of negative factors that the body and reproductive system have to cope with, such as exposure to cigarette smoke. Environmental insults during the maternal, infancy and adult phases of human development can mediate mechanisms disturbing morphologic aspects, endocrine aspects, fertilization aspects or antioxidants of testicular tissue and can have severe and irreversible effects on spermatogenesis in a subject or its offspring.

Ultimately, UMI could be caused by any number or combination of effects such as morphologic, molecular and genetic defects. Nevertheless, as the prevalence of UMI has been rapidly increasing in recent times and environmental and lifestyle factors have been drastically changing in recent years, it is not an unwarranted assumption to conclude that lifestyle factors such as smoking play at least some, if not a definitive, role in the development of UMI. As seen in this review, there is wide consensus amongst researchers that cigarette smoke with all its components combined is detrimental to an individual's health and has negative implications for reproduction and should be strongly discouraged.

Diagnoses, evaluations and treatment recommendations for male infertility and analyses of the results of studies evaluating the effects of environmental factors on the male reproductive system are, however, not without their constraints and weaknesses. ART is not the beginning and end to male infertility. It is merely a back road around the real issue. It is thus of paramount importance that research be done on UMI and the effects of environmental exposure on the reproductive system so that we may better our understanding of and eventually hope for solutions to the prevention and cure of this growing and topical problem.

Chapter 3: Materials and Methods

3.1.   Introduction

In this chapter a short overview of the parameters measured, and their significance to fertility studies, will be discussed followed by a description of the materials, methods and research protocols utilized through the duration of the study.

3.1.1. Semen Analyses: Spermatozoal Parameters

When ejaculated, semen consists of a mixture of concentrated spermatozoa, stored in the epididymis, and seminal fluid secreted by the accessory sex glands. Several characteristics are of vital importance to spermatozoal function, when exposed to the environment of the cervical mucus and vagina, while attempting to reach and fuse with egg. The total number (and concentration) of spermatozoa, their viability, motility and number of intact acrosomes are all crucial attributes that lead to the success of oocyte fertilization.[130] There is a direct correlation between: spermatozoal motility and pregnancy rates, total number of spermatozoa/concentration of spermatozoa and pregnancy rates, spermatozoal viability- and epididymal- pathology and male infertility and between disordered acrosome reactions and male infertility. These parameters are of cardinal importance in the assessment of the fertilizing ability of spermatozoa and the effects of exogenous factors such as nicotine on the fertilizing ability and function of spermatozoa.[86,135-137]

3.1.2. Antioxidant Analyses: Biochemical Parameters

As previously noted, excess ROS production and associated OS have been shown to affect spermatozoal functional parameters and testicular tissue. ROS damages spermatozoal membranes and causes impaired spermatozoal motility as well as impaired spermatozoal morphology and concentration. ROS generated by abnormal spermatozoa and leukocytes activates caspases that initiate a chain of downstream signalling reactions ultimately leading to apoptosis. Spermatozoal DNA is however protected against moderate OS insult by

antioxidants and antioxidant enzymes in the seminal plasma and by the tight packaged organization of DNA, but excess ROS still damages DNA by disruption of DNA methylation. By employing enzyme assays specific for these antioxidant reactions, the antioxidant capacity of cells or tissue can be monitored and thus when used, in addition to other markers of OS such as LPO, can be utilized as indicative markers of the antioxidant state of a specific cell or tissue.[60,138-143,132-134]

3.2.   Experimental Framework

Two treatment models were employed: animals were exposed to nicotine *in utero* while human and rat spermatozoa were exposed to nicotine *in vitro*. Semen parameters such as motility, viability and acrosome reaction were monitored in the *in vitro* group. Biochemical analyses of the tissue of the *in utero* exposure group included measurement of LPO and antioxidant enzyme activity. Figure 3.1 gives an overview of the experimental procedure followed during the study.

Effect of Nicotine Exposure

Model 1: Prenatal exposure (n=6)

Biochemical Parameters

Antioxidant Enzyme Activity

In Utero (Rat subjects)

Pre-pubertal (Day 42) (n=6)
Adolescent (Day 84) (n=6)
Adulthood (Day 168) (n=6)

Tissue Disruption (Lipid Peroxidation)

Model 2(a): Human Sperm (n=12)

Sperm Parameters

In Vitro

Motility
Viability
Concentration
Acrosome Reaction

Model 2(b): Rat Sperm (n=6 normal)

Model 2(b): Rat Sperm (n=6 obese)

Male Reproductive System

Figure 3.1: Methodological framework of the study.

3.3.   Ethical Clearance

Institutional Review Board (IRB) permission and ethical clearance was applied for and obtained, prior to the onset of the study, from the Health Research Ethics Committee (Tygerberg, South Africa).

Ethics Reference Number: S12/03/084

Federal Wide Assurance Number: 00001372

IRB Number: IRB0005239

3.4.   Treatment and Sample Collection

Model 1: *In utero* effect of prenatal nicotine exposure on the antioxidant enzyme activity and LPO of the reproductive system of adult male Wistar rats: The Stellenbosch University Animal Facility (Association for Assessment and Accreditation of Laboratory Animal Care International accredited) bred healthy Wistar rats by allowing adult male rats of ±300g to mate with adult female rats of ±200g. All rats had free access to nutritional sources and were housed at 25°C on a 12 hour day/night cycle simulating conditions in their species specific natural habitat. ±12 pregnant females (±250g) were identified, by virtue of vaginal plugs, and isolated for the study. These rats were then randomly divided into two groups: control and nicotine- each group consisting of 6 rats (Table 3.1):

Table 3.1: *In utero* treatment methodology.

Treatment Groups	Treatment	Exposure to treatment
Control (C)	Physiological saline, 0.85%, 1ml/kg/bodyweight/day	Throughout gestation & lactation
Nicotine (N)	1 mg/kg-bodyweight/day	Throughout gestation & lactation

The rats in the nicotine group were treated with a 98-100% pure solution purchased from Sigma-Aldrich ($C_{10}H_{14}N_2$; Mw= 162.2; N-3876; Lot-46H0992) (Sigma-Aldrich, St. Louis, MO, USA) diluted to the desired concentrations with phosphate buffered saline. Nicotine was administered via subcutaneous injection at a dosage of 1 mg/kg-bodyweight/day throughout gestation and lactation (roughly the concentration which a pregnant female smoker of ±70kg is exposed to when smoking 20 cigarettes per day).[144] Weaning of the rats from their mothers took place at about 21 days after birth. The rats in the control group were injected with a physiological saline (0.85% sterile filtered NaCl) that should not have an affect the parameters of the experiment, but conveys the stress of being injected as the other rats had to endure. The body weights of the dams were measured twice weekly to monitor wellbeing and to update the concentration of the nicotine injections accordingly. The dams were treated directly with nicotine throughout gestation and lactation and rat the pups were therefore exclusively exposed to the treatment via placental uptake and lactation. The day of birth was designated as day 0 in the age monitoring of the pups and rats reach adulthood at ±50/60 days after birth. After birth the body weights of the rat pups were measured and after appropriate periods of nicotine exposure [42 days- pre-pubertal (± 10 human years of age), 84 days- adolescence (± 14 human years of age), 168 days- adulthood (± 22 human years of age)],[145] 36 male rats (6 rats exposed to nicotine and 6 rats exposed to saline per age group) were sacrificed via intraperitoneal injection of Nembutal (160mg/Kg BW). The three age groups of the rats, at time of sacrifice, correlates to three stages of male development: pre-pubertal, adolescence and adulthood. The testes was excised immediately after sacrifice and, after weight recording, rinsed and 100-150mg of tissue was gently homogenized in 1.5ml of phosphate buffered assay-specific buffer in a Bullet Blender homogenizer (Next Advance, New York, USA). The tissue homogenates were then flash frozen and stored at -80^{0}C for further study of biochemical parameters.

Model 2(a): *In vitro* effect of direct nicotine exposure on spermatozoal parameters of human subjects: The study was conducted in the Stellenbosch University Reproductive Research

(SURGG) of the Division of Medical Physiology in the Faculty of Medicine and Health Sciences at Stellenbosch University. Subjects eligible for this study were fertile donors between the ages of 19 and 26 who regularly, voluntarily and anonymously donated semen samples, to the reproductive laboratory, displaying functional parameters of concentration between 12-16 $x10^6$ per ml, volume between 1.4-1.7 ml and total count between 33-46 $x10^6$ per ejaculate according to specifications supplied by the WHO guidelines to be regarded as fertile. Smoking was the only exclusion criteria for the study.[129] Exposure of donors to environmental and passive smoking was not controlled for, but the level of nicotine in circulation resulting from environmental smoke exposure is of such a low level that it is not of consequence to such a study.[75] Each volunteer signed an informed consent form for their donated samples to be used for general laboratory experiments as approved by the University's ethics committee. Consecutive donors (n=12) were approached for consent to be included in this specific study and have their samples exposed to nicotine. Semen samples were collected by masturbation into sterile containers, after sexual abstinence of 2-7 days as prescribed by the WHO.[130] After collection, human semen samples were left for 30 minutes in the incubator to liquefy at 37°C. Routine macroscopic and microscopic semen analysis was performed immediately after liquefaction and according to WHO guidelines: semen viscosity, semen appearance, semen volume, semen pH, initial motility and concentration were recorded. After the initial analyses, the semen sample underwent a double wash in HAMS F10 solution [containing 3% Bovine Serum Albumin (BSA)] to separate the motile spermatozoal fractions from the seminal fluid and other debris by centrifuging twice for 5 minutes at 1800rpm. The isolated spermatozoa were resuspended in HAMS-BSA (3%) solution and diluted to $5x10^6$ spermatozoa/ml for use in nicotine exposure procedures and semen analyses.

Model 2(b): *In vitro* effect of direct nicotine exposure on spermatozoal parameters of normal and obese Wistar rats: Rats which were used for obese part of study were bred as described above, but were fed with a high fat diet comprising of 390g normal rat chow, 100g fructose,

100g casein, 10g cholesterol and 400g holsum. After excision, normal and obese rat epididymides were cut into smaller pieces and left for 15 minutes in 5ml HAMS (containing 3% BSA) allowing the spermatozoa to swim out of the epididymis into the HAMS-BSA solution. The HAMS-BSA solution containing the isolated spermatozoa was then extracted and diluted to 5×10^6 spermatozoa/ml for use in nicotine exposure procedures and spermatozoal analyses.

Spermatozoa (rat or human) were subsequently divided into 5 aliquots of which 4 were exposed to different concentrations of nicotine and 1 aliquot served as a control. The nicotine working solutions were freshly prepared every third day and stored at 4°C in a dark glass container to prevent degradation due to light exposure. The four treatment aliquots were exposed to the following concentrations of nicotine diluted with phosphate buffered saline: 0.1mM, 1mM, 5mM, 10mM. These were of much higher quantity than that present in the seminal plasma of casual and habitual smokers, in an attempt to establish at what concentration nicotine adversely affects spermatozoal parameters. Nicotine concentrations of between 70µg/l (0.00043mM) and 300µg/l (0.00185mM) are commonly found in the semen of casual (1-10 cigarettes/day) and habitual smokers (>30 cigarettes/day), but have rarely, without controversy, been reported to affect semen parameters at these concentrations.[22-24,73-74] The aliquots were then incubated at 37°C, 5%CO_2 and 96% humidity and the analysis was performed at the following time intervals: baseline, 30min, 60min, 120min and 180min.

3.5.   Semen Analyses

3.5.1. Motility

Motility parameters (fast progressive, slow progressive, non-progressive) of at least 200 spermatozoa was assessed by computer aided sperm analysis (CASA) [as seen in Figure 3.2], using a sperm class analyser (SCA, Barcelona, Spain), sampled from 5×10^6 spermatozoa/ml suspended in HAMS-BSA solution, of the control and treatment groups,

using an eight-chamber standard count analysis slide (Leja products, GN Nieuw-Vennep, The Netherlands) at 37°C. The system analysed the total motility, progressive motility (percentage of A+B level of spermatozoa) and kinematic and velocity parameters such as curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), amplitude of lateral head displacement (ALH) and beat cross-frequency (BCF). The following CASA settings for rat spermatozoal analyses were used: Pseudo Negative phase, Ph2/3 condenser, 4x objective lens, no filter, Brightness ± 42, Contrast ± 640. CASA settings for human spermatozoa: Pseudo Positive phase, Ph1 condenser, 10x objective lens, green filter, Brightness ± 166, Contrast ± 420.[130]
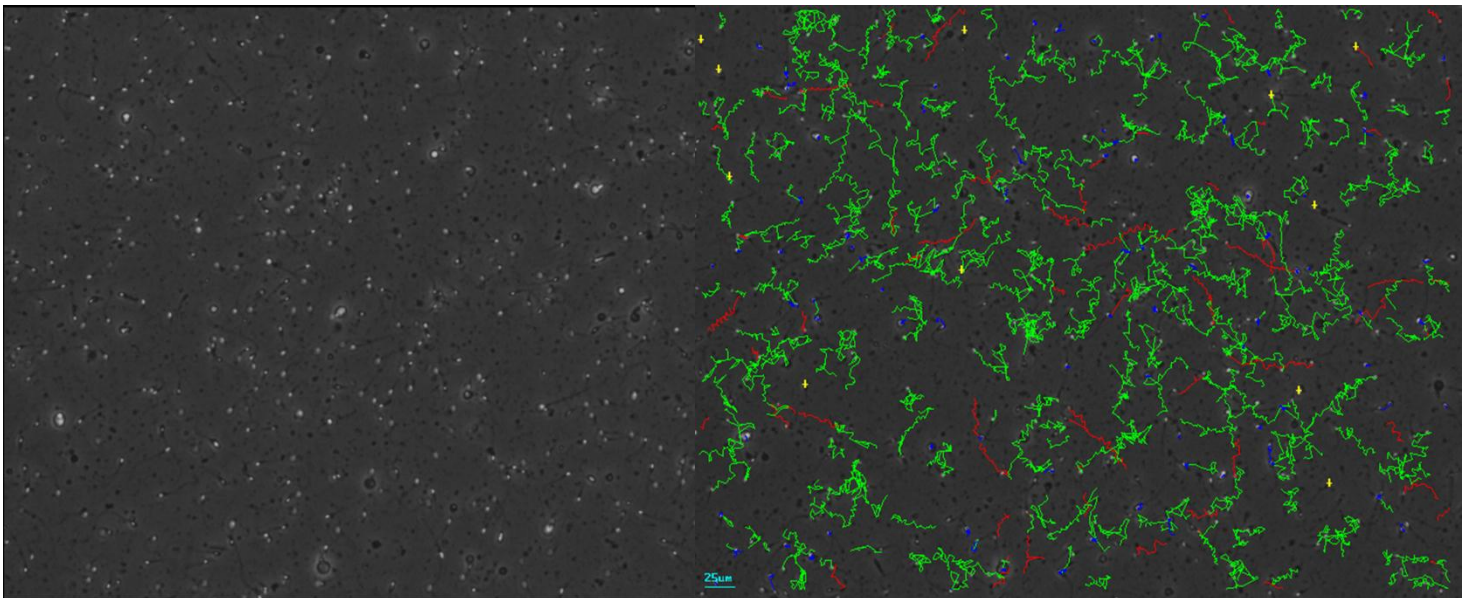
Figure 3.2: Figure illustrating a sperm population as visualized, analysed and quantified by a CASA system.

3.5.2. Viability

Viability status of spermatozoa was assessed microscopically by means of a dye exclusion staining technique (Eosin/Nigrosin) [as seen in Figure 3.3]. The dye exclusion technique is based on the principle that the plasma membranes of dead and dying cells are permeable to membrane-impermeant stains. ±10µl of a 5×10^6 spermatozoa/ml suspension was added and

mixed to ±100µl eosin and ±10µl nigrosin. A 6µl drop of the stained mixture was placed on a glass slide, smeared and allowed to air dry overnight. The slides were mounted by applying a drop of a permanent non-aqueous mounting medium and a cover slip to the slide. The viability of 100-200 spermatozoa was visually determined to be viable or non-viable under brightfield microscopy (100x). All slides were coded to be blind to treatment and were scored by one person.[130,146]
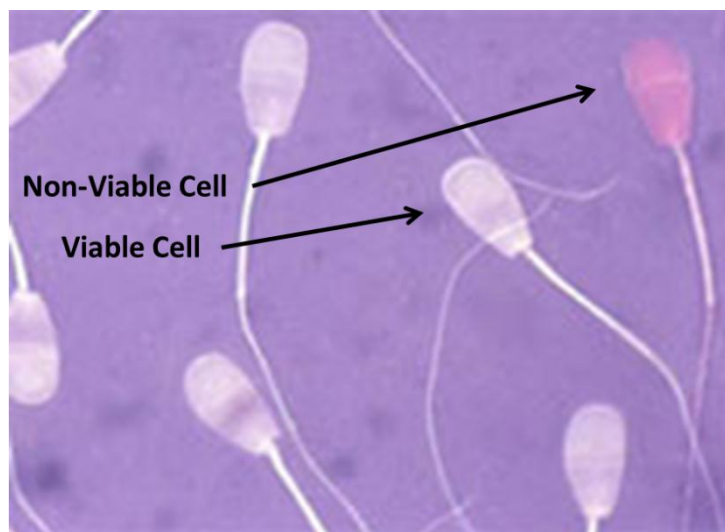
Figure 3.3: Figure illustrating a sperm population stained with eosin/nigrosin for viability.[175]

3.5.3. Acrosome Reaction

The acrosome reaction takes place at the zona pellucida after the binding of spermatozoa and can be assessed on spermatozoa removed from the surface of the zona pellucida, or exposed to disaggregated human zona pellucida proteins by visualization with fluorescently labelled lectins. The acrosomal status of treated and untreated spermatozoa was assessed microscopically. Spermatozoa were left to incubate for three hours. ±10µl of a $5x10^6$ spermatozoa/ml suspension was smeared on a glass slide, air dried, fixed with 70% ethanol (30min, 4°C) and again air dried. Smears were stained with Fluorescein Isothiocyanate-Labelled Pisum Sativum Agglutinin (FITC-PSA) [50µl FITC + 450µl phosphate buffered saline] and air dried in a dark environment for 30 minutes. Slides were then rinsed with

distilled water, exposed to DACO Antifade mounting medium (Glostrup, Denmark), covered with a cover slip and the acrosomes of 100-200 spermatozoa were visually determined to be intact or reacted (totally + partially) under fluorescence microscopy at 100x magnification (as seen in Figure 3.4). Spermatozoa with bright green fluorescence at the acrosome region were counted as acrosome intact while those with pale green fluorescing regions were counted as acrosome reacted. All slides were coded to be blind to treatment and were be scored by one person.[130,137]
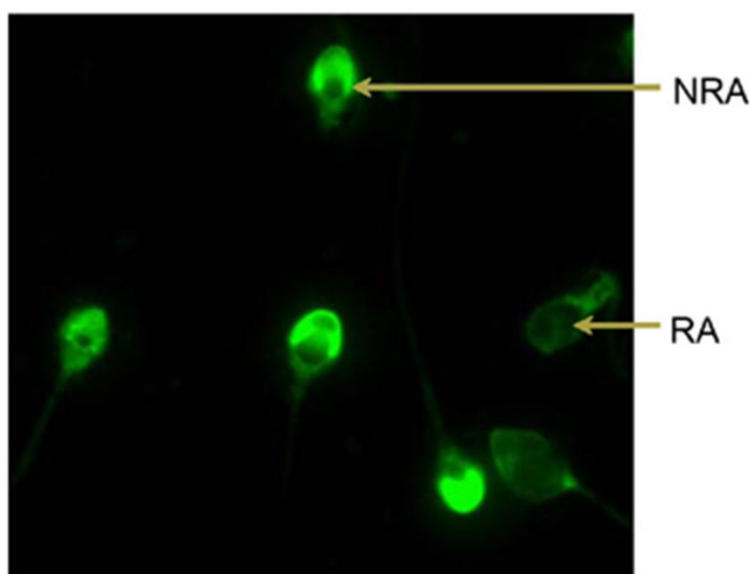
Figure 3.4: Figure illustrating a sperm population probed with FITC-PSA for acrosomal status.[176] NRA= Non-reacted acrosome. RA= Reacted acrosome.

3.6.   Biochemical Parameters

3.6.1. Protein Determination

Protein determination was done on all tissue homogenates in order to quantitatively express all biochemical parameter-units as a function of amount of protein. The Bicinchoninic Acid (BCA) Protein Assay determines protein concentration by the formation of a Cu^{2+}-protein complex under alkaline conditions and the reduction of this complex to Cu^{1+} which is

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proportionate to the amount of protein present in the sample. Protein determination was done by means of a plate reader (FLUOstar Omega, BMG Labtech, Offenburg, Germany) [as seen in Figure 3.5] and BCA Protein Assay Kit (cat# BCA1, Sigma-Aldrich, St. Louis, MO, USA) in accordance to the assay protocol provided by the manufacturer. The assay was initiated by adding 200µl of BCA working reagent to 25µl standard/sample in triplicate on a flat bottom 96-well plate. The plate was sealed and incubated at 37°C for 30 minutes, allowed to cool for 15min after which the absorbance was read at 562nm. The protein concentration was determined by comparison of absorbance to the standard curve and expressed as mg/ml protein.[147]

Figure 3.5: Figure illustrating a FLUOstar Omega plate reader as used for all biochemical analyses.[177]

3.6.2. Superoxide Dismutase

SOD activity can be measured from the reaction of xanthine oxidase and hypoxanthine, in the presence of a tetrazolium salt, and the subsequent production of superoxide (O_2^-). Tetrazolium salt is converted to a coloured product: formazan dye which is quantitatively

analysable by a plate reader with absorbance capacity. The SOD activity of the sample is then determined by calculating the percentage inhibition of the rate of formazan formation. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. The activity of SOD in testicular tissue was determined by means of a plate reader and the SOD (cat#706002, Cayman, Ann Arbor, MI, USA) assay kit and was run in accordance to the assay protocol provided by the manufacturer. The reaction was initialized by the addition of 20µl of xanthine oxidase to 200µl radical detector and 10µl sample/standard (in triplicate); with the whole mixture then being incubated for 20 minutes and the absorbance read at 450nm at room temperature. The SOD activity was expressed as Units/mg of tissue.

3.6.3. Catalase

CAT activity can be measured by monitoring the peroxidatic function of CAT. CAT reacts with methanol in the presence of an optimal concentration of H_2O_2. The formaldehyde produced can be colourmetrically measured by its ability to form bicyclic heterocycles with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald). One CAT unit is defined as the amount of enzyme required to decompose 1.0mmole of hydrogen peroxide (10.3mM) per minute at pH 7.0 and 25°C. The activities of CAT in testicular tissue was determined by means of a plate reader and the CAT (cat# 707002, Cayman, Ann Arbor, MI, USA) assay kit and was run in accordance to the assay protocol provided by the manufacturer. The reaction initiated when 100µl of assay buffer was added to 30µl methanol and 20µl of sample/standard (in triplicate) and 20µl H_2O_2 and the mixture incubated on a shaker at room temperature for 20 minutes. The reaction terminated when 30µl of diluted Potassium Hydroxide was added to each well and the reaction visualisation took place by adding 30µl Purpald to each well while incubating the plate for a further 10 minutes. 10µl of Potassium Periodate was added to each well and the plate incubated on a shaker for 5 minutes and the absorbance was read at 540nm using a plate reader. The CAT activity was expressed as Units/mg tissue.[148]

3.6.4. Glutathione

Determination of the activity of GSH is based on the GSH mediated conversion of a luciferin derivative into luciferin in the presence of glutathione S-transferase (GST). The amount of GSH present in the sample is proportionate to the signal created by the firefly luciferase-coupled reaction. The GSH level was measured by using a GSH Assay kit (GSH-Glo™) and was done in accordance to the assay protocol as provided by the manufacturer (cat# V6911, Promega, Madison, WI, USA). The reaction was initiated by adding 50µl GSH-Glo™ 2x Reagent to 50µl tissue/standard (in triplicate) on a flat-bottom 96-well plate and allowed to incubate at room temperature for 30 minutes. 100µl of luciferin detection reagent was added to each well of the 96 well microplate (as seen in Figure 3.6) incubated along with the tissue mixture on a shaker for 15 minutes before assessing the luminescence in a plate reader. GSH activity was expressed as µM GSH/mg protein.
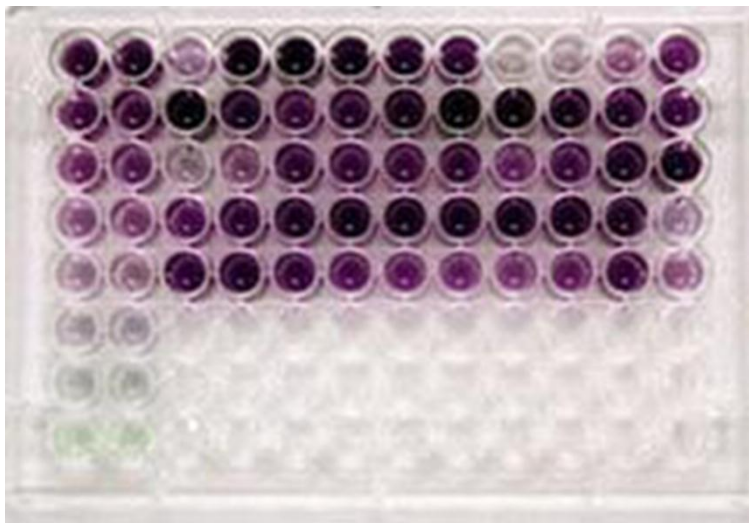
Figure 3.6: Figure illustrating a 96 well plate as used for all biochemical protocols and analyses.[178]

3.6.5. Lipid Peroxidation

LPO is a well-established mechanism of antioxidant dysfunction and damage in cells. Lipid peroxides are unstable products of OS in cells. Complex lipid peroxides such as

Malondialdehyde (MDA) are natural by-products of LPO. LPO was measured by monitoring the direct expression of thiobarbituric acid reactive substance (TBARS): MDA. The activity was determined by means of a plate reader and the OxiSelect TBARS (cat# STA-330, Cell Biolabs, San Diego, USA) assay kit and was run in accordance to the assay protocol provided by the manufacturer. The reaction was initiated by adding 150µl sample/standard to 150µl SDS Lysis solution and 375µl TBA Reagent to 1.5ml eppendorf reaction tubes. The tubes were incubated at 95°C for 45-60min. The vials were brought down to room temperature in an ice bath for 5min and then centrifuged for 15 minutes at 3000rpm. The supernatant of the reaction mixture was removed and 150µl was transferred (in triplicate) to a black fluorescence 96-well plate and the fluorescence was read at an excitation wavelength of 540nm and an emission wavelength of 590nm. LPO is expressed as µM MDA/mg protein.[149]

3.7.   Statistical Analyses

Data was analysed using the appropriate process associated with the general linear model procedure according to the latest version of Statistica (Oklahoma, USA) software. The associated pairwise adjustment parameter was used to determine whether the means of the respective parameters differed statistically and significantly. Values were considered statistically significant if $P < 0.05$. All data is expressed as mean ± standard error mean (SEM).

Chapter 4: Results

The results obtained during the course of the investigation are provided in this chapter. Results are presented in the form of bar graphs (See Figures 4.1-4.17) displaying the SEM and the significance where applicable, followed by a description of statistically significant results. The tables containing the measurements for all biochemical- and spermatozoal – parameters captured by the SCA® or quantified by the enzyme assay kits during the investigation which are relevant to the graphs discussed are presented at the end of each subsection (See Tables 4.3-4.13). The complete spermatozoal analyses performed for the *in vitro* part of the study is attached as Addendum A. Although statistical analyses for the full set of *in vitro* spermatozoal parameters have been performed and provided in the addendum, due to the scope of the study not all parameters will form part of the discussion in this dissertation, and have merely been included for the reader's interest and to provide information that may prove to be useful in future investigations. Those parameters which are accompanied by bar graphs will form part of the main discussion.

4.1   Model 1: Effect of *In Utero* Nicotine Exposure on Antioxidant Enzyme Activity and Lipid Peroxidation of the Reproductive System of Adult Male Wistar Rats

In order to investigate the effect of *in utero* nicotine exposure on the antioxidant enzyme activity and LPO of the testicular tissue from adult male Wistar rats, the differences in various antioxidant enzyme levels were examined of tissue from rats all exposed to nicotine or physiologic saline *in utero* and sacrificed at different ages (42 days, 84 days and 168 days). The aim in this section of the study was to identify any possible differences in antioxidant levels between the control and treated rats and between the respective age groups (see Figures 4.1-4.7 and Tables 4.1-4.2).

4.1.1. Protein Concentration

Results indicated that younger age groups displayed significantly higher testes protein concentrations than the older age groups (day 42>84>168) as seen in Figure 4.1. Protein concentration of control groups was significantly higher at younger ages (C42 vs. C84: 5.900±0.273 mg/ml vs. 4.238±0.094 mg/ml; $p<0.05$; C42 vs. C168: 5.900±0.273 mg/ml vs. 3.369±0.082 mg/ml; $p<0.05$; C84 vs. C168: 4.238±0.094 mg/ml vs. 3.369±0.082 mg/ml; $p<0.05$). Both N84 and N168 groups were significantly lower than N42 group (N42 vs. N84: 6.374±0.431 mg/ml vs. 4.585±0.122 mg/ml; $p<0.05$; N42 vs. N168: 6.374±0.431 mg/ml vs. 4.443±0.070 mg/ml; $p<0.05$). Protein concentrations of all treated groups displayed higher values than control groups (N>C). The N168 group was however, the only group significantly higher than its relevant control group (N168 vs. C168: 4.443±0.070 mg/ml vs. 3.369±0.082 mg/ml; $p<0.05$).

Protein	Control 42	Nicotine 42	Control 84	Nicotine 84	Control 168	Nicotine 168
Protein	5.900213	6.373815	4.237932	4.584759	3.369283	4.442623

n=6.　a= $p<0.05$ vs. own control group
b= $p<0.05$ vs. C42 group
c= $p<0.05$ vs. C84 group
d= $p<0.05$ vs. N42 group
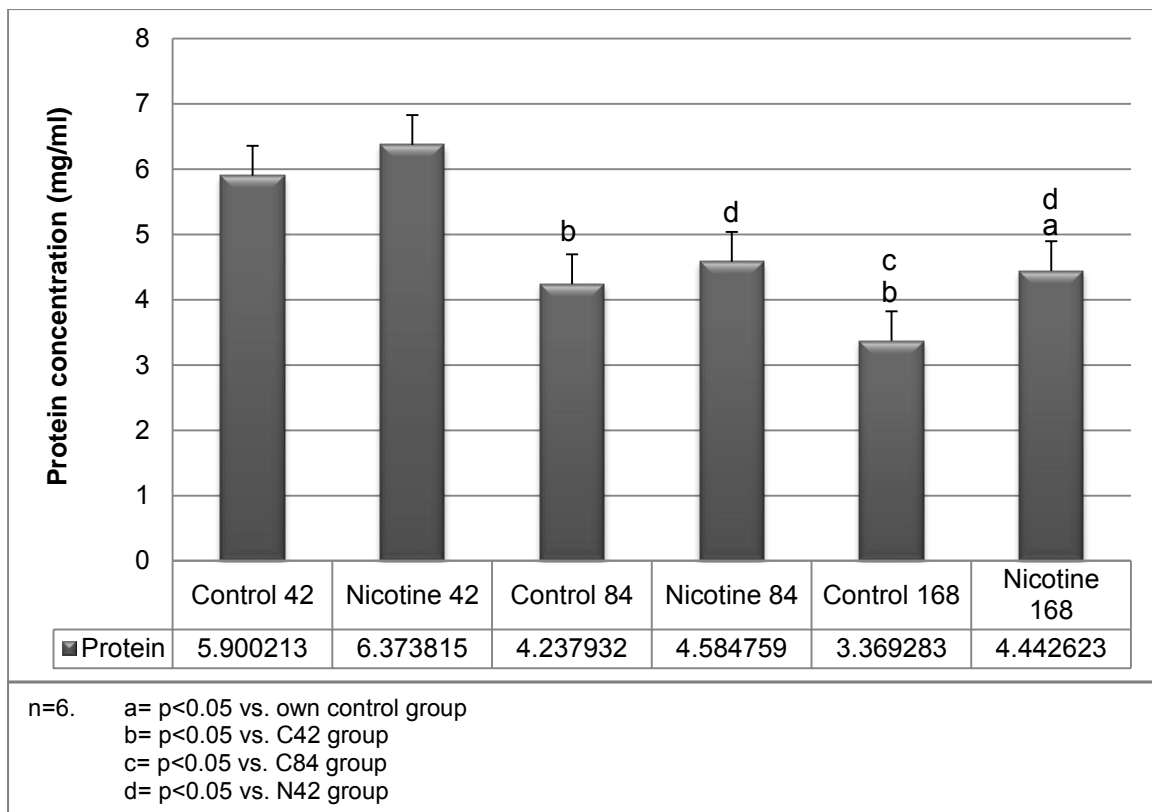
Figure 4.1: Effect of *in utero* nicotine- and control -treatment on testicular protein concentration of male Wistar rat offspring of increasing age (day 42, 84, 168).

4.1.2. Superoxide Dismutase

Results indicated that older age groups displayed significantly higher SOD concentrations compared to the younger age groups (day 42<84<168) as seen in Figure 4.2. SOD concentration of all groups (treated and non-treated) was significantly higher at older ages (C42 vs. C84: 14.928±0.864 Units/mg protein vs. 38.137±1.009 Units/mg protein; $p<0.05$; C42 vs. C168: 14.928±0.864 Units/mg protein vs. 63.475±1.719 Units/mg protein; $p<0.05$; C84 vs. C168: 38.137±1.009 vs. 63.475±1.719 Units/mg protein; $p<0.05$) (N42 vs. N84: 13.338±0.316 Units/mg protein vs. 35.075±1.274 Units/mg protein; $p<0.05$; N42 vs. N168: 13.338±0.316 Units/mg protein vs. 45.653±0.644 Units/mg protein; $p<0.05$; N84 vs. N168: 35.075±1.274 Units/mg protein vs. 45.653±0.644 Units/mg protein; $p<0.05$). SOD concentrations of all treated groups displayed lower, however not significantly lower, values than control groups (N<C). The N168 group was however, the only group significantly lower than its relevant control group (N168 vs. C168: 45.653±0.644 Units/mg protein vs. 63.475±1.719 Units/mg protein; $p<0.05$).
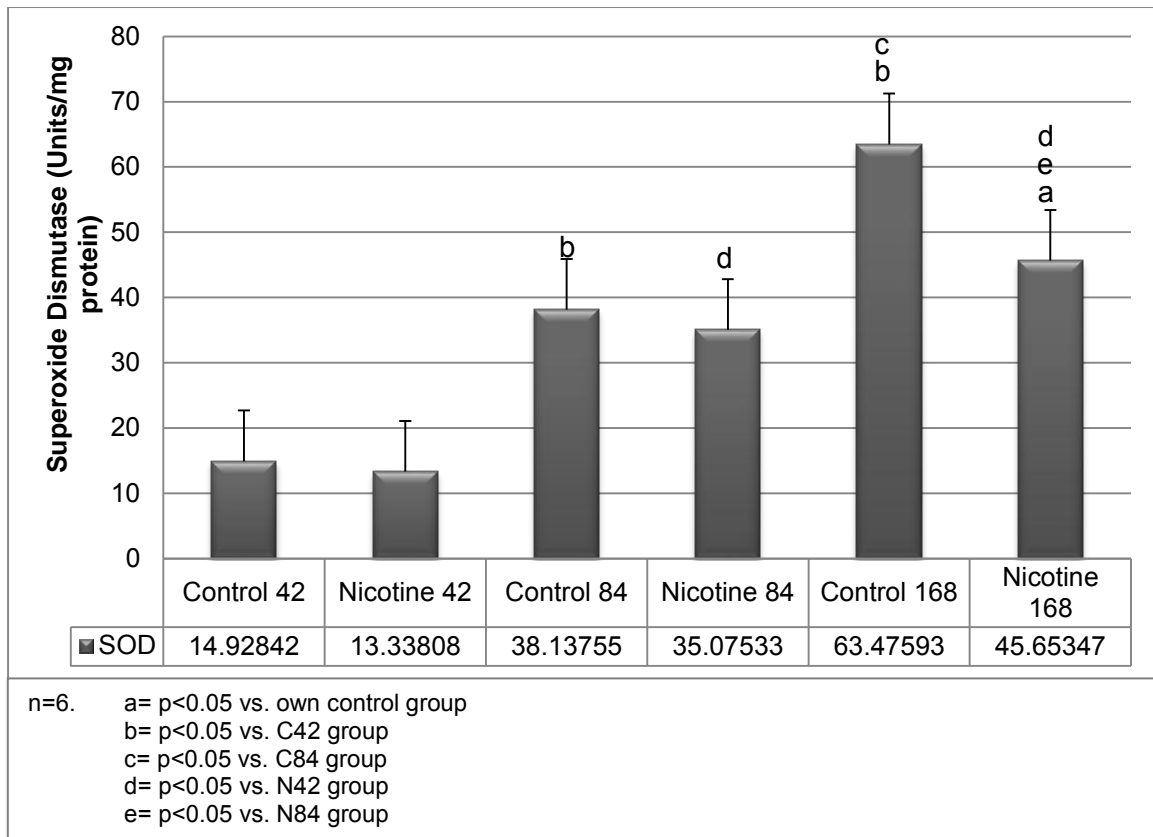
Figure 4.2: Effect of *in utero* nicotine- and control -treatment on testicular SOD levels of male Wistar rat offspring of increasing age (day 42, 84, 168) (n=6).

4.1.3. Catalase

Results indicated that older age groups displayed significantly higher CAT concentrations than the younger age groups (day 42<84<168) as seen in Figure 4.3. CAT concentration of control groups was significantly higher at older ages (C42 vs. C84: 20.961±0.704 Units/mg protein vs. 32.547±2.452 Units/mg protein; $p<0.05$; C42 vs. C168: 20.961±0.704 Units/mg protein vs. 52.265±0.613 Units/mg protein; $p<0.05$; C84 vs. C168: 32.547±2.452 Units/mg protein vs. 52.265±0.613 Units/mg protein; $p<0.05$). Both N84 and N168 groups were significantly higher than N42 group (N42 vs. N84: 19.596±1.309 Units/mg protein vs. 34.739±0.558 Units/mg protein; $p<0.05$; N42 vs. N168: 19.596±1.309 Units/mg protein vs. 35.499±3.049 Units/mg protein; $p<0.05$). CAT concentrations of N42 and N84 groups displayed similar values to the control groups (N=C). The N168 group was, however, the

only group significantly lower than its relevant control group (N168 vs. C168: 35.499±3.049 Units/mg protein vs. 52.265±0.613 Units/mg protein; p<0.05).
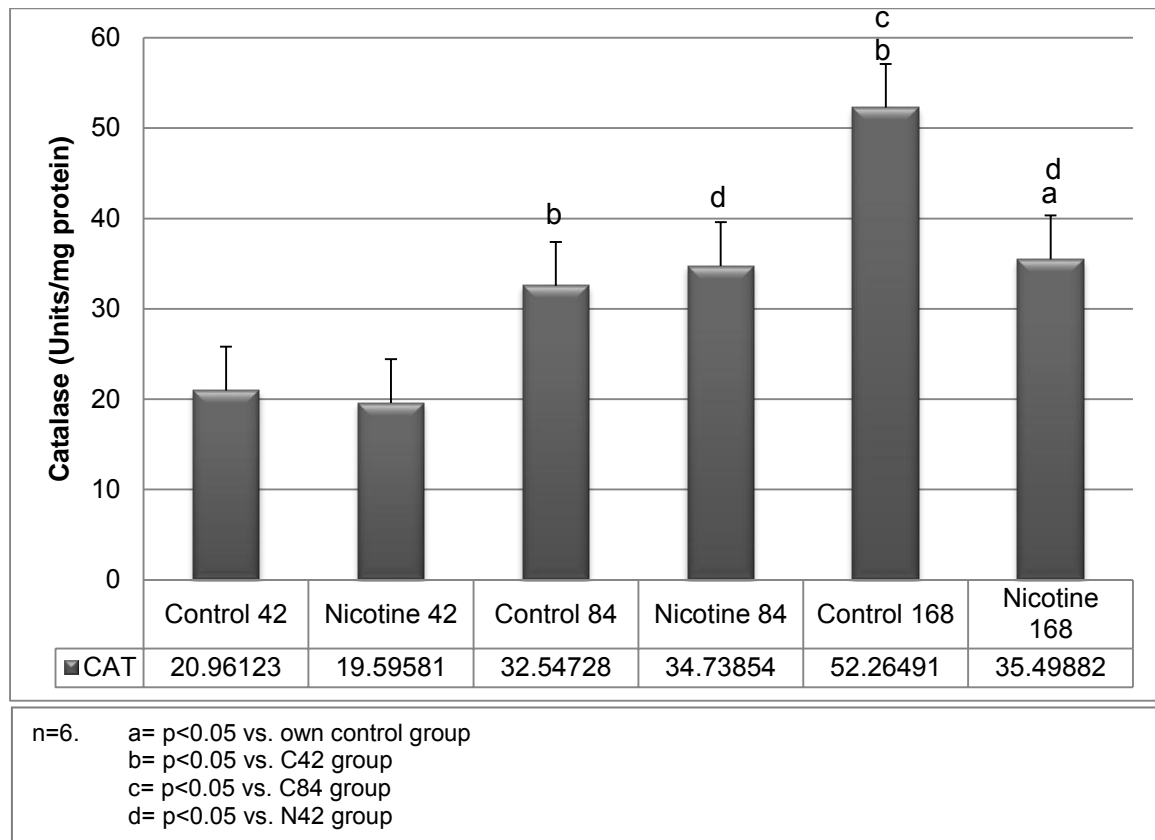
Figure 4.3: Effect of *in utero* nicotine- and control -treatment on testicular CAT levels of male Wistar rat offspring of increasing age (day 42, 84, 168) (n=6).

4.1.4. Glutathione

Results indicated that older age groups displayed higher, yet not significantly higher, GSH concentrations than the younger age groups (day 42<84<168) as seen in Figure 4.4. GSH concentration of C168 group was significantly higher than C42 group (C42 vs. C168: 1074.331±40.781 Units/mg protein vs. 1367.485±88.179 Units/mg protein; p<0.05). GSH concentrations of N42 and N84 groups displayed similarly to their respective control groups (N=C). The N168 group was however, the only group significantly lower than its relevant control group (C168 vs. N168: 1367.485±88.179 Units/mg protein vs. 1170.577±59.635 Units/mg protein; p<0.05).
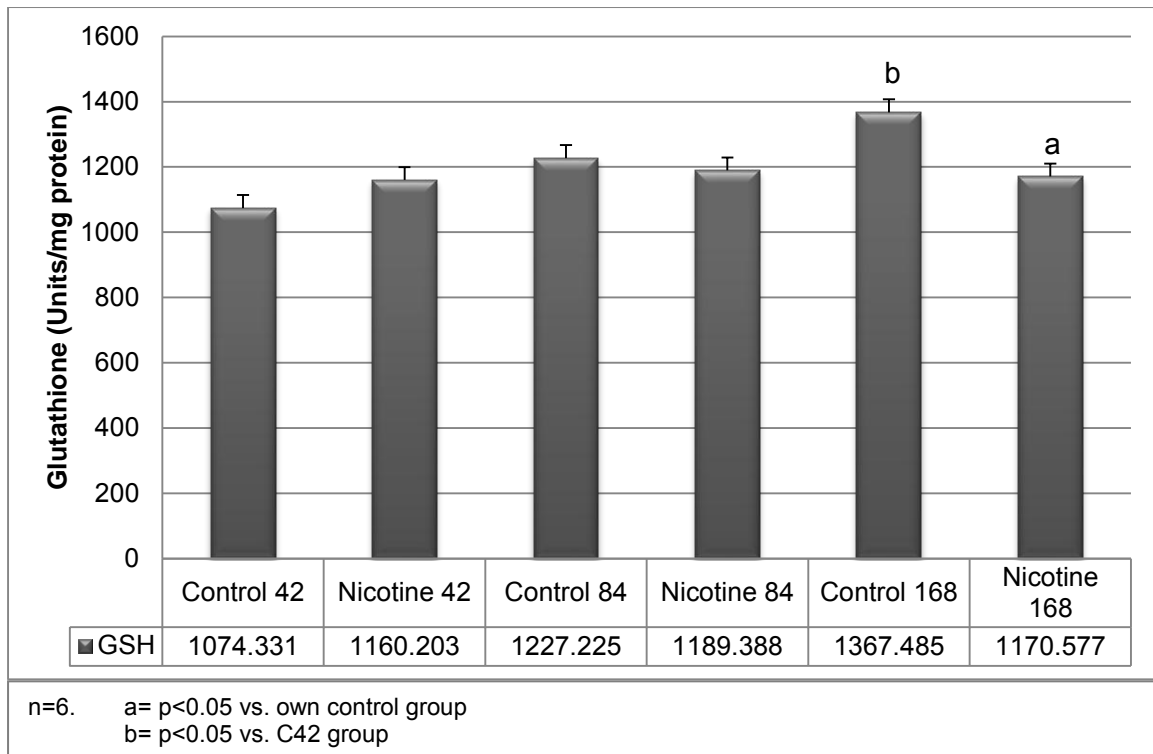
Figure 4.4: Effect of *in utero* nicotine- and control -treatment on testicular GSH levels of male Wistar rat offspring of increasing age (day 42, 84, 168) (n=6).

4.1.5. Lipid Peroxidation

Results reflected that the d42 and d168 age groups displayed significantly higher MDA concentrations than the d84 group (day 42<84,168) as seen in Figure 4.5. MDA concentrations of C42 and C168 were comparable, while both significantly higher than the C84 group (C42 vs. C84: 7.036±0.299 Units/mg protein vs. 2.883±0.163 Units/mg protein; $p<0.05$; C168 vs. C84: 6.674±0.233 Units/mg protein vs. 2.883±0.163 Units/mg protein; $p<0.05$). The N168 group was significantly higher than the N84 and N42 groups, while the N42 group was significantly higher than the N84 group. (N42 vs. N84: 5.024±0.726 Units/mg protein vs. 2.502±0.196 Units/mg protein; $p<0.05$; N42 vs. N168: 5.024±0.726 Units/mg protein vs. 6.469±0.484 Units/mg protein; $p<0.05$; N84 vs. N168: 2.502±0.196 Units/mg protein vs. 6.469±0.484 Units/mg protein; $p<0.05$). MDA concentrations of N84 and N168 groups displayed similarly to the control groups (N=C). The N42 group was however, the

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only group significantly lower than its relevant control group (N42 vs. C42: 5.024±0.726 Units/mg protein vs. 7.036±0.299 Units/mg protein; p<0.05).
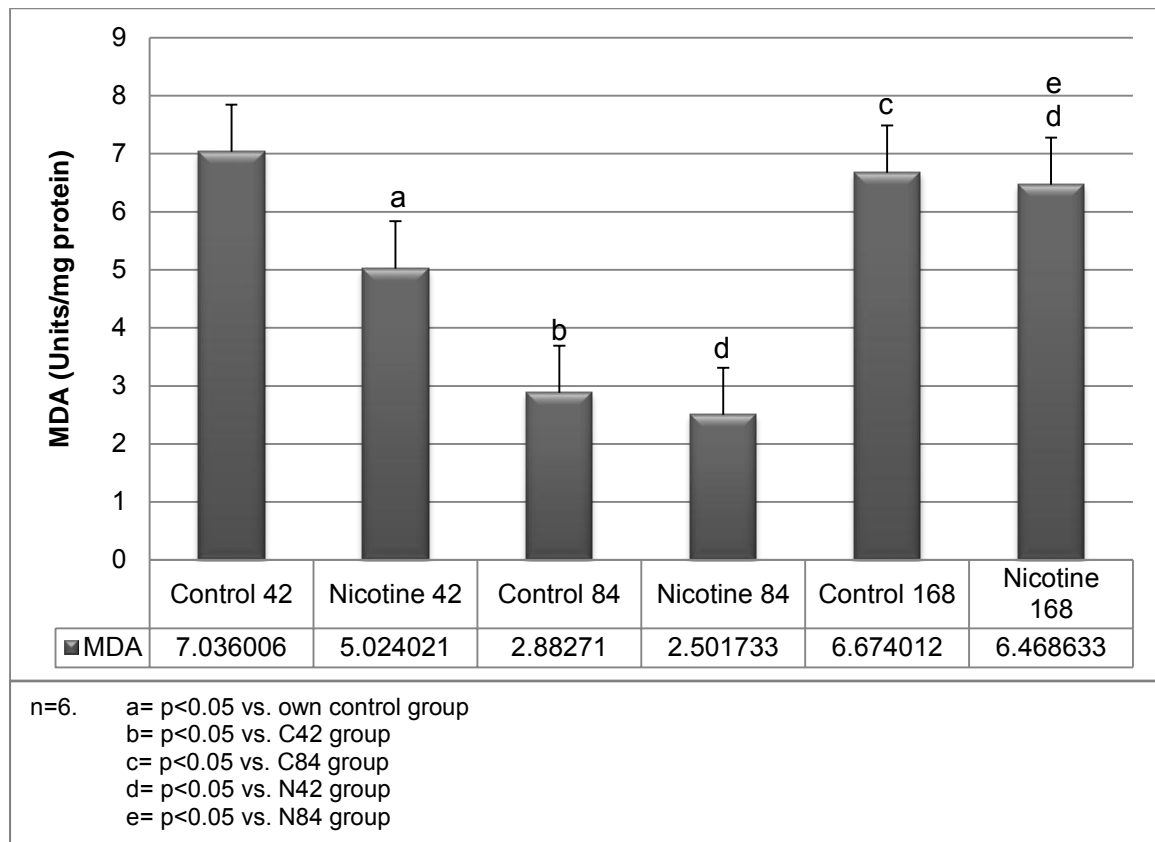
Figure 4.5: Effect of *in utero* nicotine- and control -treatment on testicular LPO of male Wistar rat offspring of increasing age (day 42, 84, 168) (n=6).

4.1.6. Treatment Significance

Results of totalled age groups indicate that protein concentration was significantly less for the control group than for the treatment group (C vs. N: 4.502±0.271 Units/mg protein vs. 5.133±0.256 Units/mg protein; p<0.05) as seen in Figure 4.6 and Table 4.1. For all other antioxidant enzymes and LPO markers the control group values were significantly higher than the treatment group values, besides GSH, for which the control group values were higher but not significantly (CSOD vs. NSOD: 38.847±4.856 Units/mg protein vs. 31.356±3.295 Units/mg protein; p<0.05; CCAT vs. NCAT: 35.258±3.240 Units/mg protein vs.

29.944±2.065 Units/mg protein; p<0.05; CMDA vs. NMDA: 5.531±0.474 Units/mg protein vs. 4.792±0.496 Units/mg protein; p<0.05).

	Protein	SOD	CAT	GSH (x100)	MDA
Control	4.502476	38.8473	35.25781	12.23013	5.530909
Nicotine	5.133732	31.35563	29.94439	11.7339	4.792035

n=18.   a= p<0.05 vs. own control group
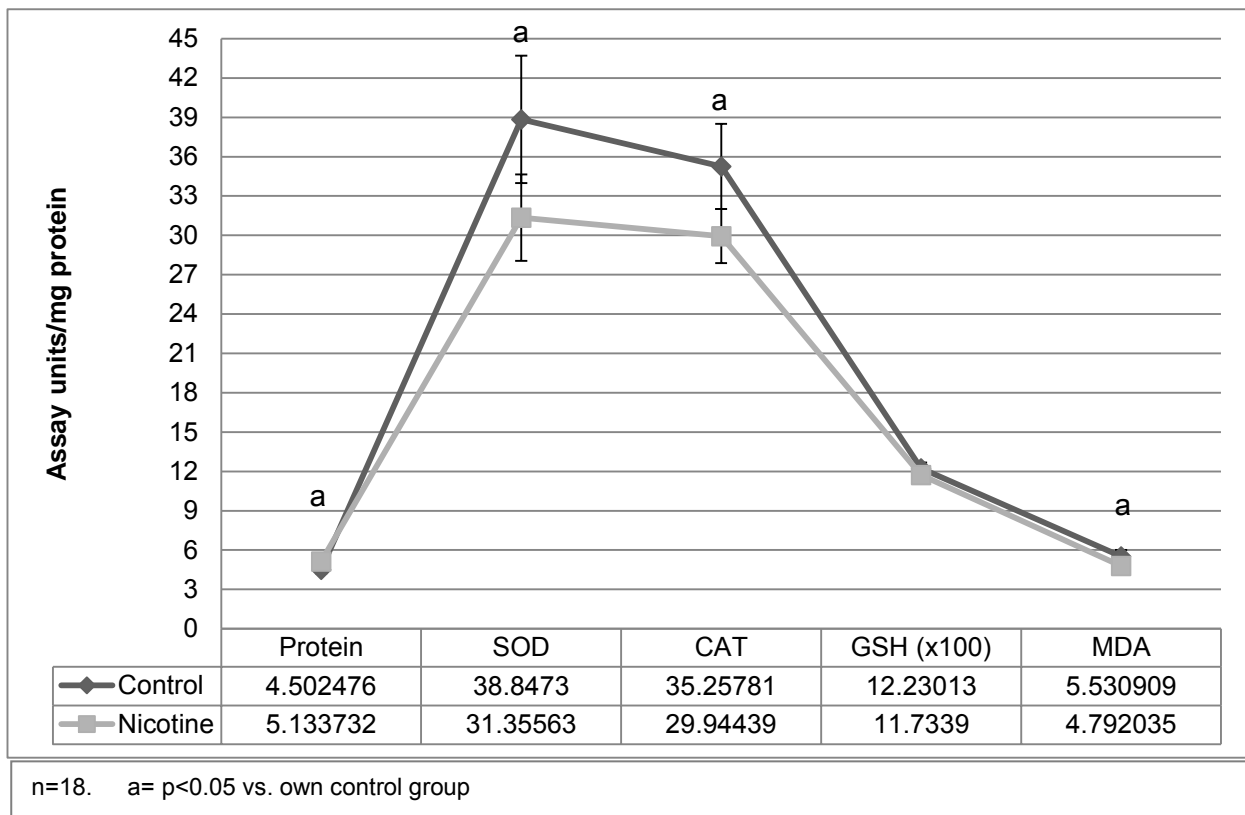
Figure 4.6: Effect of *in utero* nicotine exposure vs. control treatment on protein concentrations, antioxidant enzymes and LPO of male Wistar rat offspring (n=6 for all groups).

Table 4.1: Effect of *in utero* nicotine exposure vs. control treatment on protein concentrations, antioxidant enzymes and LPO of male Wistar rat offspring.

Total	[Protein]	SOD	CAT	GSH	MDA
Control	4.502476± 0.271495	38.84730± 4.856353	35.25781± 3.240067	1223.013± 44.07592	5.530909± 0.473572
Nicotine	5.133732± 0.256044[a]	31.35563± 3.294670[a]	29.94439± 2.065294[a]	1173.390± 35.41421	4.792035± 0.496339[a]

n=18.   a= p<0.05 vs. own control group

4.1.7. Age Significance

Results of totalled treatment groups indicates that protein concentration was significantly lower for the older age groups (d42 vs. d84: 6.137±0.253 mg/ml vs. 4.411±0.090 mg/ml; $p<0.05$; d42 vs. d168: 6.137±0.253 mg/ml vs. 3.906±0.17 mg/ml; $p<0.05$; d84 vs. d168: 4.411±0.090 mg/ml vs. 3.906±0.17 mg/ml; $p<0.05$) as seen in Figure 4.7 and Table 4.2. For all other antioxidant enzymes the older age groups were significantly higher than the younger age groups, except for the LPO markers which were comparable for the d42 and d168 group and significantly lower for the d84 group (SOD42 vs. SOD84: 14.133±0.5 Units/mg protein vs. 36.606±0.902 Units/mg protein; $p<0.05$; SOD42 vs. SOD168: 14.133±0.5 Units/mg protein vs. 54.565±2.826 Units/mg protein; $p<0.05$; SOD84 vs. SOD168: 36.606±0.902 Units/mg protein vs. 54.565±2.826 Units/mg protein; $p<0.05$) (CAT42 vs. CAT84: 20.279±0.738 Units/mg protein vs. 33.643±1.24 Units/mg protein; $p<0.05$; CAT42 vs. CAT168: 20.279±0.738 Units/mg protein vs. 43.882±2.930 Units/mg protein; $p<0.05$; CAT84 vs. CAT168: 33.643±1.24 Units/mg protein vs. 43.882±2.930 Units/mg protein; $p<0.05$) (GSH42 vs. GSH84: 1117.267±46.611 Units/mg protein vs. 1208.306±29.811 Units/mg protein; $p<0.05$; GSH42 vs. GSH168: 1117.267±46.611 Units/mg protein vs. 1269.031±58.793 Units/mg protein; $p<0.05$; GSH84 vs. GSH168: 1208.306±29.811 Units/mg protein vs. 1269.031±58.793 Units/mg protein; $p<0.05$) (MDA42 vs. MDA84: 6.030±0.482 Units/mg protein vs. 2.71±0.134 Units/mg protein; $p<0.05$; MDA42 vs. MDA168: 6.030±0.482 Units/mg protein vs. 6.571±0.258 Units/mg protein; $p<0.05$; MDA84 vs. MDA168: 2.71±0.134 Units/mg protein vs. 6.571±0.258 Units/mg protein; $p<0.05$).

	Protein	SOD	CAT	GSH (x100)	MDA
Day 42	6.137014	14.13325	20.27852	11.17267	6.030014
Day 84	4.411345	36.60644	33.64291	12.08306	2.709539
Day 168	3.905953	54.5647	43.88186	12.69031	6.571323

n=18.   a= p<0.05 vs. own parameter day 42 group
b= p<0.05 vs. own parameter day 84 group
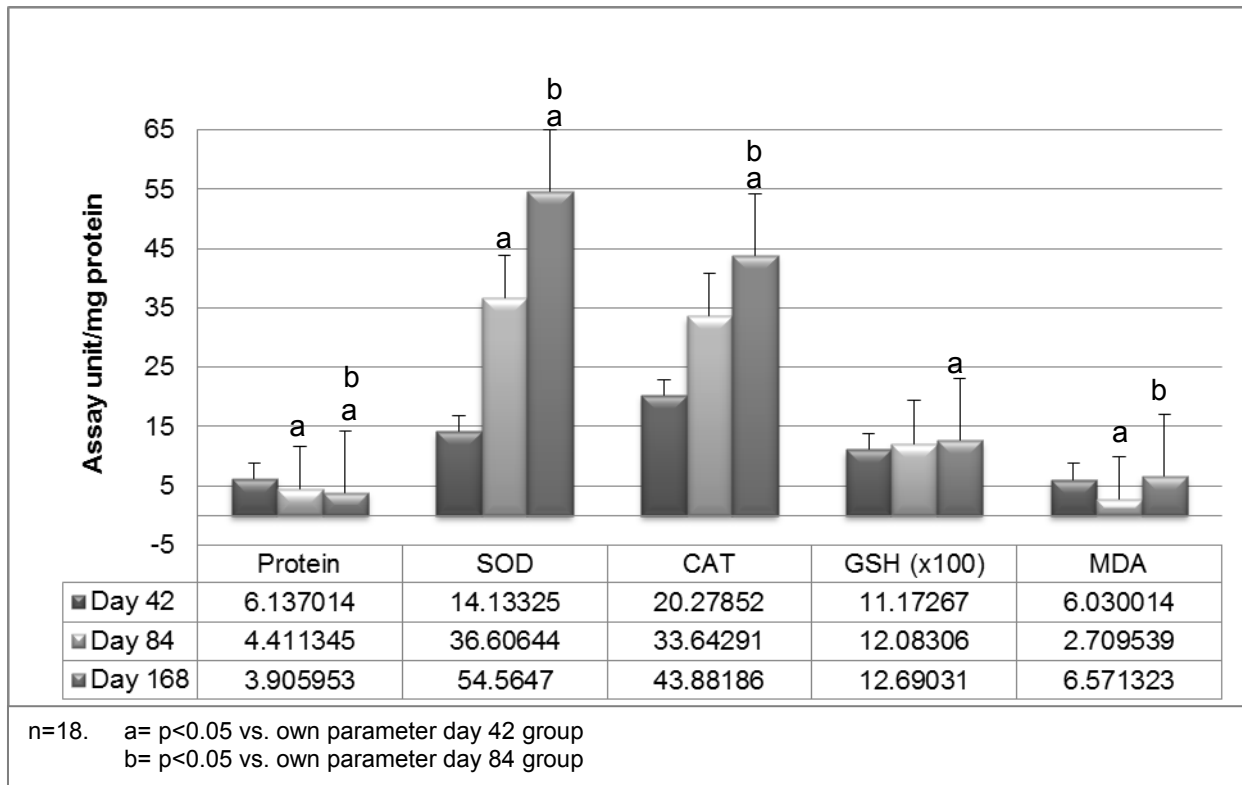
Figure 4.7: Effect of increasing age (day 42, 84, 168) on protein concentrations, antioxidant enzymes and LPO of male Wistar rat offspring (n=6 for all groups).

Table 4.2: Effect of increasing age (day 42, 84, 168) on protein concentrations, antioxidant enzymes and LPO of male Wistar rat offspring.

Total	Protein [ ]	SOD	CAT	GSH	MDA
42 day	6.137014± 0.253486	14.13325± 0.499966	20.27852± 0.737807	1117.267± 46.61100	6.030014± 0.481640
84 day	4.411345± 0.090048[a]	36.60644± 0.902061[a]	33.64291± 1.243444	1208.306± 29.81146	2.709539± 0.133542[a]
168 day	3.905953± 0.169814[ab]	54.56470± 2.825852[ab]	43.88186± 2.930407[ab]	1269.031± 58.79314[a]	6.571323± 0.258088[b]

n=18.   a= p<0.05 vs. own parameter day 42 group
b= p<0.05 vs. own parameter day 84 group

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4.1.8. Biochemical Parameter Table

The table below (Table 4.3) summarizes all means of biochemical parameters measured of testicular tissue of 36 male Wistar rats (n=6 per group) subjected to nicotine exposure or control treatment *in utero*.

Table 4.3: Effect of *in utero* nicotine exposure vs. control treatment on protein concentrations, antioxidant enzymes and LPO of male Wistar rat offspring of different ages (day 42, 84,168).

	[Protein]	SOD	CAT	GSH	MDA
42 Control	5.900213± 0.273332	14.92842± 0.864143	20.96123± 0.703852	1074.331± 40.78092	7.036006± 0.299110
42 Nicotine	6.373815± 0.430791	13.33808± 0.316533	19.59581± 1.308945	1160.203± 84.61015	5.024021± 0.725551[a]
84 Control	4.237932± 0.093944[b]	38.13755± 1.009154[b]	32.54728± 2.451951[b]	1227.225± 42.16087	2.882710± 0.163178[b]
84 Nicotine	4.584759± 0.121751[d]	35.07533± 1.274453[d]	34.73854± 0.557538[d]	1189.388± 44.60587	2.501733± 0.195837[d]
168 Control	3.369283± 0.082133[bc]	63.47593± 1.719347[bc]	52.26491± 0.613463[bc]	1367.485± 88.17891[b]	6.674012± 0.233388[c]
168 Nicotine	4.442623± 0.070214[ad]	45.65347± 0.644811[ade]	35.49882± 3.049188[ad]	1170.577± 59.63545[a]	6.468633± 0.484143[de]

n=6. a= p<0.05 vs. own age group and parameter control group
b= p<0.05 vs. own parameter C42 group
c= p<0.05 vs. own parameter C84 group
d= p<0.05 vs. own parameter N42 group
e= p<0.05 vs. own parameter N84 group

4.2.   Model 2: *In Vitro* Effect of Direct Nicotine Exposure on the

Spermatozoal Parameters of Human and Rat Subjects

In order to investigate the effect of *in vitro* nicotine exposure on the spermatozoal parameters of humans, Wistar rats and obese Wistar rats, the differences in various spermatozoal parameters were examined by use of semen processed by a double wash separation technique. These spermatozoal parameters were all measured using the different staining and probing techniques as specified by the WHO. The aim in this section of the study was to identify any possible differences in spermatozoal parameters between the control and treated spermatozoa of humans, obese and normal rats (See Figures 4.8-4.17 and Tables 4.4-4.13).

4.2.1. Model 2(a): *In Vitro* Effect of Direct Nicotine Exposure on the
Spermatozoal Parameters of Human Subjects

4.2.1.1.   Total Motility

Results indicated that there were no indicative trends pertaining to increasing concentrations of nicotine or increasing time periods of measurement after nicotine exposure and influence on percentage total motile human spermatozoa as seen in Figure 4.8. The 120min (after exposure) group did however show a significant relation to increasing concentrations of nicotine and decrease in percentage total motile human spermatozoa (30, 60, 180min > 120min [1,5,10mM]). The percentage total motility of the 1*120, 5*120 and 10*120 groups were all significantly less than the C*120 group (C*120 vs. 1*120: 68.292±5.845 % vs. 57.950±5.845 %; $p<0.05$; C*120 vs. 5*120: 68.292±5.845 % vs. 49.083±5.845 %; $p<0.05$; C*120 vs. 10*120: 68.292±5.845 % vs. 47.067±5.845 %; $p<0.05$). The percentage total motility of the 5*120 and 10*120 groups was also significantly decreased as opposed to the 0.1*120 group (0.1*120 vs. 5*120: 63.983±5.845 % vs. 49.083±5.845 %; $p<0.05$; 0.1*120 vs. 10*120: 63.983±5.845 % vs. 47.067±5.845 %; $p<0.05$). The percentage total motility of the 5*120 group was also significantly less than that of the 5*60 group (5*120 vs. 5*60:

49.083±5.845 % vs. 61.867±5.845 %; p<0.05). The 10*120 group percentage total motility was significantly decreased as opposed to the 1*120, 10*30, 10*60 and 10*180 groups respectively (10*120 vs. 1*120: 47.067±5.845 % vs. 57.950±5.845 %; p<0.05; 10*120 vs. 10*30: 47.067±5.845 % vs. 61.483±5.845 %; p<0.05; 10*120 vs. 10*60: 47.067±5.845 % vs. 60.858±5.845 %; p<0.05; 10*120 vs. 10*180: 47.067±5.845 % vs. 58.217±5.845 %; p<0.05). All the other percentage total motile results were relatively comparable, besides the C*120 group that proved to be significantly increased to the C*180 group (C*120 vs. C*180: 68.292±5.845 % vs. 56.517±5.845 %; p<0.05).

	C	0.1	1	5	10
■30	57.94167	58.58333	63.23333	58.68333	61.48333
■60	64.625	61.2	58.46667	61.86667	60.85833
■120	68.29167	63.98333	57.95	49.08333	47.06667
■180	56.51667	61.78333	57.475	54.275	58.21667

n=12.   a= p<0.05 vs. C*120 group
         b= p<0.05 vs. 0.1*120 group
         c= p<0.05 vs. 1*120 group
         d= p<0.05 vs. 5*60 group
         e= p<0.05 vs. 10*30 group
         f= p<0.05 vs. 10*60 group
         g= p<0.05 vs. 10*120 group

Figure 4.8: Effect of *in vitro* nicotine administration (at different concentrations: 0.1, 1, 5, 10 mM) on total motility of human spermatozoal total motility at increasing time points after exposure (30, 60, 120, 180 min) (n=12).

4.2.1.2.   Progressive Motility

Results indicated that the progressive motility values of the 30min and 60min groups remained similar as nicotine concentrations increased, while the progressive motility of the 120min and 180min groups significantly decreased as nicotine concentrations increased (30, 60min> 120,180min[1,5,10mM]) as seen in Figure 4.9. The 120min (after exposure) group did however show a significant relation to increasing concentrations of nicotine and decreases in progressive motility in human spermatozoa. The progressive motility of the 1*120, 5*120 and 10*120 groups were all significantly less than that of the C*120 group and 0.1*120 group (C*120 vs. 1*120: 47.642±5.982 % vs. 29.192±5.982 %; $p<0.05$; C*120 vs. 5*120: 47.642±5.982 % vs. 23.567±5.982 %; $p<0.05$; C*120 vs. 10*120: 47.642±5.982 % vs. 20.658±5.982 %; $p<0.05$; 0.1*120 vs. 1*120: 40.308±5.982 % vs. 29.192±5.982 %; $p<0.05$; 0.1*120 vs. 5*120: 40.308±5.982 % vs. 23.567±5.982 %; $p<0.05$; 0.1*120 vs. 10*120: 40.308±5.982 % vs. 20.658±5.982 %; $p<0.05$). The C*120 progressive motility group also proved to be significantly higher than the C*30 and C*180 groups (C*120 vs. C*30: 47.642±5.982 % vs. 34.825±5.982 %; $p<0.05$; C*120 vs. C*180: 47.642±5.982 % vs. 33.867±5.982 %; $p<0.05$). The 1*120 group's progressive motility proved to be significantly less than that of the 1*60 group (1*120 vs. 1*60: 29.192±5.982 % vs. 41.067±5.982 %; $p<0.05$). The 5*120 group's progressive motility proved to be significantly less than that of the 5*30 and 5*60 groups (5*120 vs. 5*30: 23.567±5.982 % vs. 36.742±5.982 %; $p<0.05$; 5*120 vs. 5*60: 23.567±5.982 % vs. 41.133±5.982 %; $p<0.05$). The 10*120 group's progressive motility proved to be significantly less than that of the 10*30 and 10*60 groups (10*120 vs. 10*30: 20.658±5.982 % vs. 38.075±5.982 %; $p<0.05$; 10*120 vs. 10*60: 20.658±5.982 % vs. 37.942±5.982 %; $p<0.05$). The progressive motility of the 5*180 group was significantly lower than the 5*60 group (5*180 vs. 5*60: 28.392±5.982 % vs. 41.133±5.982 %; $p<0.05$).

	C	0.1	1	5	10
■ 30	34.825	39.24167	39.25833	36.74167	38.075
■ 60	45.00833	42.775	41.06667	41.13333	37.94167
■ 120	47.64167	40.30833	29.19167	23.56667	20.65833
■ 180	33.86667	33.76667	29.9	28.39167	27.06667

n=12.   a= p<0.05 vs. C*30 group
b= p<0.05 vs. C*120 group
c= p<0.05 vs. 0.1*120 group`
d= p<0.05 vs. 1*60 group
e= p<0.05 vs. 5*30 group
f= p<0.05 vs. 5*60 group
g= p<0.05 vs. 10*30 group
h= p<0.05 vs. 10*60 group

Figure 4.9: Effect of *in vitro* nicotine administration (at different concentrations: 0.1, 1, 5, 10 mM) on progressive motility of human spermatozoa at increasing time points after exposure (30, 60, 120, 180 min) (n=12).

4.2.1.3.   Viability

Results indicated that the viability of human spermatozoa decreased as nicotine concentrations and time after exposure increased (30> 60> 120>180min; C>0.1>1>5>10mM]) as seen in Figure 4.10. The viability C*60, C*120, C*180, 5*30 and 10*30 groups were all significantly lower than that of the C*30 group (C*30 vs. C*60: 60.549±3.532 % vs. 49.057±3.532 %; p<0.05; C*30 vs. C*120: 60.549±3.532 % vs. 46.187±3.532 %; p<0.05; C*30 vs. C*180: 60.549±3.532 % vs. 47.473±3.532 %; p<0.05; C*30 vs. 5*30: 60.549±3.532 % vs. 48.962±3.532 %; p<0.05; C*30 vs. 10*30: 60.549±3.532 % vs. 44.571±3.537 %; p<0.05). The viability of the 1*180, 5*180 and 10*180 groups were significantly lower than that of the C*180 group (C*180 vs. 1*180: 47.473±3.532 % vs.

40.006±3.532 %; p<0.05; C*180 vs. 5*180: 47.473±3.532 % vs. 40.370±3.602 %; p<0.05; C*180 vs. 10*180: 47.473±3.532 % vs. 36.628±3.532 %; p<0.05). The viability of the 0.1*120, 0.1*180, 5*30 and 10*30 groups were all significantly less than that of the 0.1*30 group (0.1*30 vs. 0.1*120: 55.438±3.532 % vs. 44.642±3.532 %; p<0.05; 0.1*30 vs. 0.1*180: 55.438±3.532 % vs. 43.534±3.532 %; p<0.05; 0.1*30 vs. 5*30: 55.438±3.532 % vs. 48.962±3.532 %; p<0.05; 0.1*30 vs. 10*30: 55.438±3.532 % vs. 44.571±3.532 %; p<0.05). The 1*60 group viability also proved to be significantly lower than the viability of the 0.1*60 group (1*60 vs. 0.1*60: 49.537±3.532 % vs. 43.334±3.532 %; p<0.05). The viability of the 1*60, 1*120, 1*180 and 10*30 groups all presented significantly lower than that of the 1*30 group (1*30 vs. 1*60: 54.812±3.532 % vs. 43.334±3.532 %; p<0.05; 1*30 vs. 1*120: 54.812±3.532 % vs. 47.123±3.532 %; p<0.05; 1*30 vs. 1*180: 54.812±3.532 % vs. 40.006±3.532 %; p<0.05; 1*30 vs. 10*30: 54.812±3.532 % vs. 44.571±3.532 %; p<0.05). The viability of the 5*60 group was significantly higher than that of the 1*60 group (5*60 vs. 1*60: 49.813±3.532 % vs. 43.334±3.532 %; p<0.05). The viability of the 5*180 group was significantly lower than that of the 5*30 and 5*60 groups (5*180 vs. 5*30: 40.370±3.620 % vs. 48.962±3.532 %; p<0.05; 5*180 vs. 5*60: 40.370±3.620 % vs. 49.813±3.532 %; p<0.05). The 10*180 group's viability was also significantly lower than that of the 0.1*180, 10*30 and 10*60 groups (10*180 vs. 0.1*180: 36.628±3.532 % vs. 43.534±3.532 %; p<0.05; 10*180 vs. 10*30: 36.628±3.532 % vs. 44.571±3.532 %; p<0.05; 10*180 vs. 10*60: 36.628±3.532 % vs. 45.960±3.532 %; p<0.05).

	C	0.1	1	5	10
■30	60.5494	55.43819	54.81168	48.96248	44.57123
■60	49.05713	49.53746	43.3337	49.81304	45.96033
■120	46.18718	44.64163	47.12282	44.6713	42.8117
■180	47.47336	43.53425	40.00631	40.36963	36.62823

n=12.   a= p<0.05 vs. C*30 group
          b= p<0.05 vs. C*180 group
          c= p<0.05 vs. 0.1*30 group
          d= p<0.05 vs. 0.1*60 group
          e= p<0.05 vs. 0.1*180 group
          f= p<0.05 vs. 1*30 group
          g= p<0.05 vs. 1*60 group
          h= p<0.05 vs. 5*30 group
          i= p<0.05 vs. 5*60 group
          j= p<0.05 vs. 10*30 group
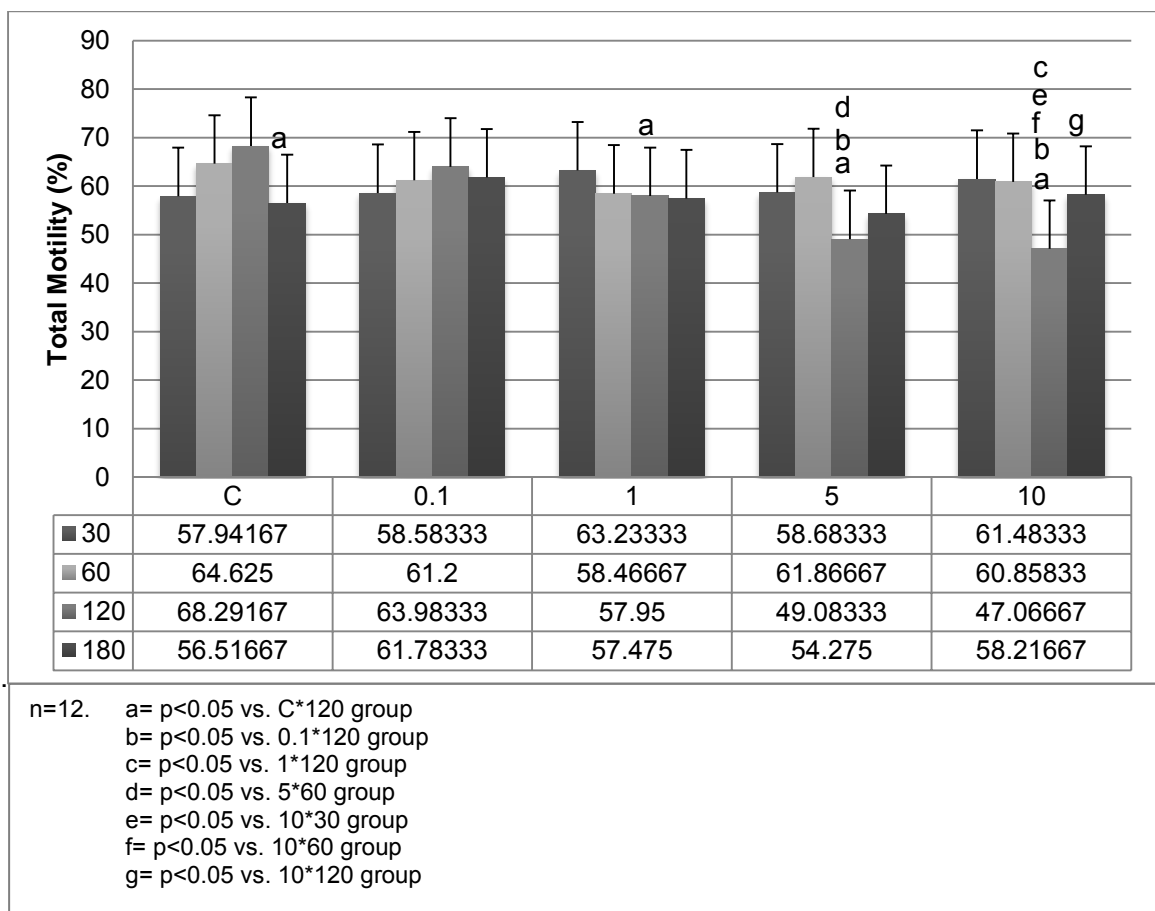   k= p<0.05 vs. 10*60 group

Figure 4.10: Effect of *in vitro* nicotine administration (at different concentrations: 0.1, 1, 5, 10 mM) on percentage viability of human spermatozoa at increasing time points after exposure (30, 60, 120, 180 min) (n=12).

4.2.1.4.   Acrosome Reaction

Results indicated that the percentage of acrosome reacted human spermatozoa increased as nicotine concentrations and time after exposure increased (30<60<120<180min; C<0.1<1<5<10mM]) as seen in Figure 4.11. The acrosome reacted cells were significantly higher for the C*120 and C*180 groups than for the C*30 group, while the 1*30 group had a significantly lower percentage acrosome reacted cells than the C*30 group (C*30 vs. C*120:

53.890±3.959 % vs. 68.283±3.959 %; p<0.05; C*30 vs. C*180: 53.890±3.959 % vs. 72.605±3.959 %; p<0.05; C*30 vs. 1*30: 53.890±3.959 % vs. 40.715±3.959 %; p<0.05). There were significantly more acrosome reacted cells in the C*120, C*180 and 10*60 groups than in the C*60 group (C*60 vs. C*120: 52.393±3.959 % vs. 68.283±3.959 %; p<0.05; C*60 vs. C*180: 52.393±3.959 % vs. 72.605±3.959 %; p<0.05; C*60 vs. 10*60: 52.393±3.959 % vs. 64.577±3.959 %; p<0.05). The acrosome reacted cells of the 0.1*120 and 0.1*180 were significantly more than that of the 0.1*30 group and the acrosome reacted cells were significantly less of the 1*30 as opposed to the 0.1*30 group (0.1*30 vs. 0.1*120: 51.280±3.959 % vs. 62.519±3.959 %; p<0.05; 0.1*30 vs. 0.1*180: 51.280±3.959 % vs. 65.952±3.959 %; p<0.05; 0.1*30 vs. 1*30: 51.280±3.959 % vs. 40.715±3.959 %; p<0.05). The 0.1*180 and 10*60 group acrosome reacted cells also proved to be significantly more than that of the 0.1*60 group (0.1*60 vs. 0.1*180: 54.853±3.959 % vs. 65.952±3.959 %; p<0.05; 0.1*60 vs. 10*60: 54.853±3.959 % vs. 64.57703±3.959 %; p<0.05). The percentage acrosome reacted cells of the 10*120 group was significantly higher than that of the 0.1*120 group (0.1*120 vs. 10*120: 62.519±3.959 % vs. 70.824±3.959 %; p<0.05). The acrosome reacted cells of the 1*60, 1*120, 1*180, 5*30 and 10*30 groups all presented significantly higher than that of the 1*30 group (1*30 vs. 1*60: 40.715±3.959 % vs. 56.494±3.959 %; p<0.05; 1*30 vs. 1*120: 40.715±3.959 % vs. 65.011±3.959 %; p<0.05; 1*30 vs. 1*180: 40.715±3.959 % vs. 69.616±3.959 %; p<0.05; 1*30 vs. 5*30: 40.715±3.959 % vs. 50.439±3.959 %; p<0.05; 1*30 vs. 10*30: 40.715±3.959 % vs. 58.308±3.959 %; p<0.05). The percentage acrosome reacted cells of the 1*180 group was significantly higher than that of the 1*60 group (1*60 vs. 1*180: 56.494±3.959 % vs. 69.616±3.959 %; p<0.05). The percentage acrosome reacted cells of the 5*120 and 5*180 groups was significantly higher than that of the 5*30 group (5*30 vs. 5*120: 50.439±3.959 % vs. 66.270±3.959 %; p<0.05; 5*30 vs. 5*180: 50.439±3.959 % vs. 71.801±3.959 %; p<0.05). The percentage acrosome reacted cells of the 5*180 group was significantly more than that of the 5*60 group (5*60 vs. 5*180: 59.224±3.959 % vs. 71.810±3.959 %; p<0.05). The acrosome reacted cells of the 10*120 and 10*180 groups presented significantly higher than that of the 10*30 group

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(10*30 vs. 10*120: 58.308±3.959 % vs. 70.824±3.959 %; p<0.05; 10*30 vs. 10*180:

58.308±3.959 % vs. 73.292±3.959 %; p<0.05;).

	C	0.1	1	5	10
30	53.89046	51.28017	40.71463	50.4388	58.30764
60	52.39318	54.85325	56.4936	59.22364	64.57703
120	68.28274	62.5188	65.01059	66.2695	70.82364
180	72.60488	65.95232	69.61596	71.8099	73.29175

n=12.　a= p<0.05 vs. C*30 group
b= p<0.05 vs. C*60 group
c= p<0.05 vs. 0.1*30 group
d= p<0.05 vs. 0.1*60 group
e= p<0.05 vs. 0.1*120 group
f= p<0.05 vs. 1*30 group
g= p<0.05 vs. 1*60 group
h= p<0.05 vs. 5*30 group
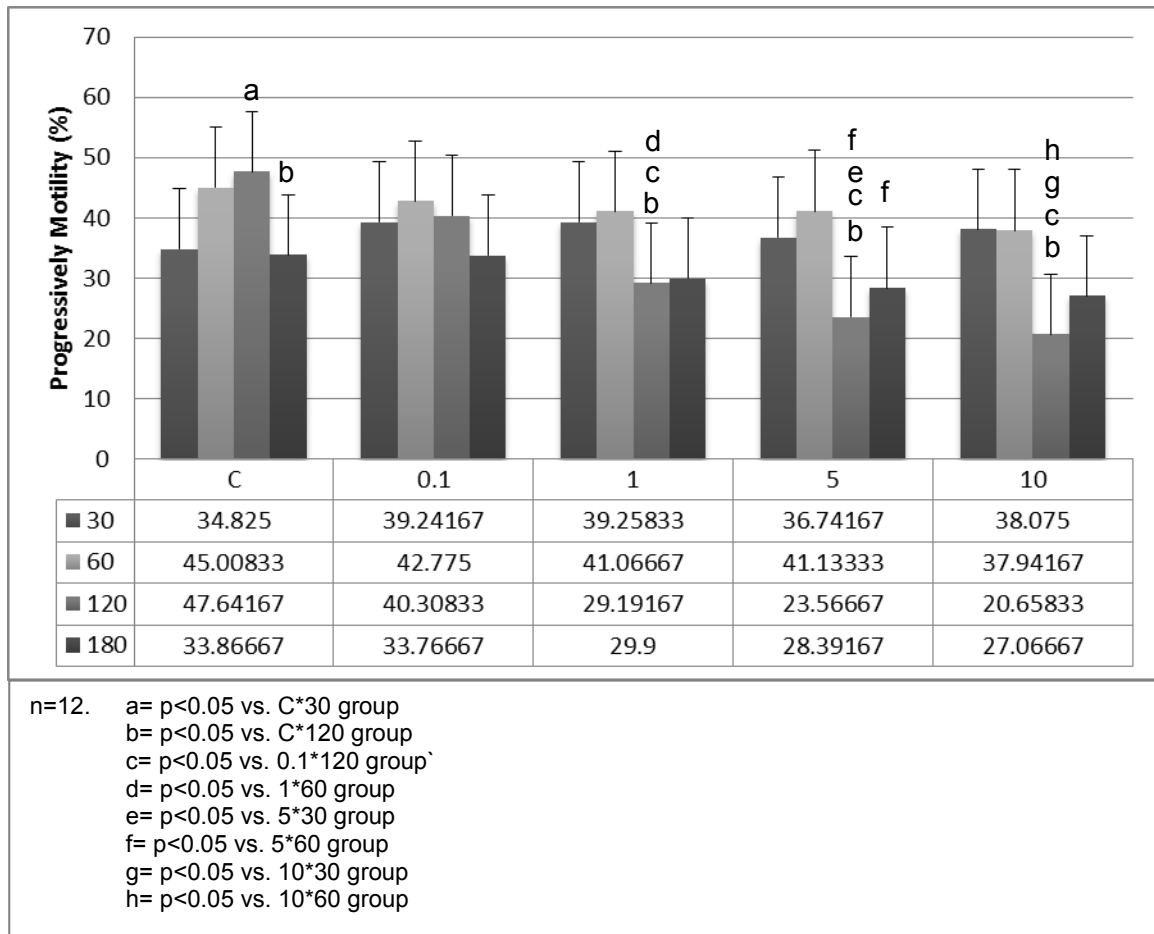i= p<0.05 vs. 5*60 group
j= p<0.05 vs. 10*30 group

Figure 4.11: Effect of *in vitro* nicotine administration (at different concentrations: 0.1, 1, 5, 10 mM) on acrosomal status of human spermatozoa (acrosome reacted %) at increasing time points after exposure (30, 60, 120, 180 min) (n=12).

4.2.2. Human Spermatozoal Parameter Tables

The tables below (Tables 4.4-4.7) summarize all means of spermatozoal kinematic parameters (discussed in previous sections) of semen of human donors (n=12) subjected to nicotine exposure at different concentrations (C, 0.1, 1, 5, 10 mM) measured at different timepoints (30, 60, 120, 180 min). For tables containing data of all parameters measured and relevant significance see Addendum A.

Table 4.4: Effect of *in vitro* nicotine administration (at different concentrations: 0.1, 1, 5, 10 mM) on total motility of human spermatozoa at increasing time points after exposure (30, 60, 120, 180 min) (n=12).

Total Motility (%)				
	Timepoints (Min)			
	30	**60**	**120**	**180**
C	57.94167± 5.844525	64.62500± 5.844525	68.29167± 5.844525	56.51667± 5.844525[a]
0.1	58.58333± 5.844525	61.20000± 5.844525	63.98333± 5.844525	61.78333± 5.844525
1	63.23333± 5.844525	58.46667± 5.844525	57.95000± 5.844525[a]	57.47500± 5.844525
5	58.68333± 5.844525	61.86667± 5.844525	49.08333± 5.844525[abd]	54.27500± 5.844525
10	61.48333± 5.844525	60.85833± 5.844525	47.06667± 5.844525[abcef]	58.21667± 5.844525[g]

n=12.   a= p<0.05 vs. C*120 group
b= p<0.05 vs. 0.1*120 group
c= p<0.05 vs. 1*120 group
d= p<0.05 vs. 5*60 group
e= p<0.05 vs. 10*30 group
f= p<0.05 vs. 10*60 group
g= p<0.05 vs. 10*120 group

Table 4.5: Effect of *in vitro* nicotine administration (at different concentrations: 0.1, 1, 5, 10 mM) on progressive motility of human spermatozoa at increasing time points after exposure (30, 60, 120, 180 min) (n=12).

Progressive Motility (%)				
		Timepoints (Min)		
Nicotine (mM)		30	60	120	180
	C	34.82500± 5.982389	45.00833± 5.982389	47.64167± 5.982389[a]	33.86667± 5.982389[b]
	0.1	39.24167± 5.982389	42.77500± 5.982389	40.30833± 5.982389	33.76667± 5.982389
	1	39.25833± 5.982389	41.06667± 5.982389	29.19167± 5.982389[bcd]	29.90000± 5.982389
	5	36.74167± 5.982389	41.13333± 5.982389	23.56667± 5.982389[bcef]	28.39167± 5.982389[f]
	10	38.07500± 5.982389	37.94167± 5.982389	20.65833± 5.982389[bcgh]	27.06667± 5.982389

n=12.   a= p<0.05 vs. C*30 group
b= p<0.05 vs. C*120 group
c= p<0.05 vs. 0.1*120 group
d= p<0.05 vs. 1*60 group
e= p<0.05 vs. 5*30 group
f= p<0.05 vs. 5*60 group
g= p<0.05 vs. 10*30 group
h= p<0.05 vs. 10*60 group

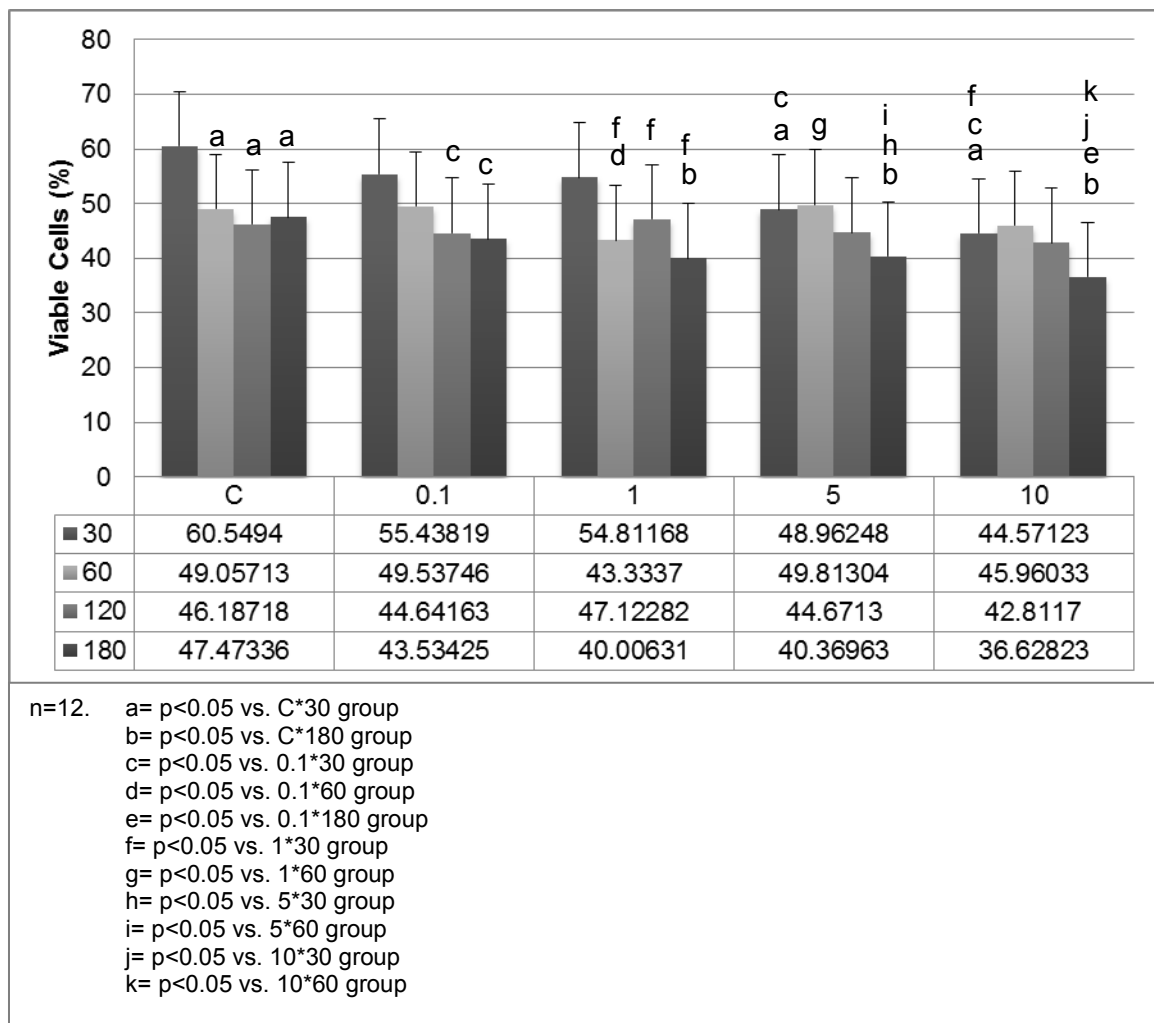**Table 4.6: Effect of *in vitro* nicotine administration (at different concentrations: 0.1, 1, 5, 10 mM) on percentage of viable human spermatozoa at increasing time points after exposure (30, 60, 120, 180 min) (n=12).**

Viable Cells (%)				
		Timepoints (Min)		
Nicotine (mM)		30	60	120	180
	C	60.54940± 3.531854	49.05713± 3.531854[a]	46.18718± 3.531854[a]	47.47336± 3.531854[a]
	0.1	55.43819± 3.531854	49.53746± 3.531854	44.64163± 3.531854[c]	43.53425± 3.531854[c]
	1	54.81168± 3.531854	43.33370± 3.531854[df]	47.12282± 3.531854[f]	40.00631± 3.531854[bf]
	5	48.96248± 3.531854[ac]	49.81304± 3.531854[g]	44.67130± 3.531854	40.36963± 3.601975[bhi]
	10	44.57123± 3.531854[acf]	45.96033± 3.531854	42.81170± 3.531854	36.62823± 3.531854[bejk]

n=12.   a= p<0.05 vs. C*30 group
b= p<0.05 vs. C*180 group
c= p<0.05 vs. 0.1*30 group
d= p<0.05 vs. 0.1*60 group
e= p<0.05 vs. 0.1*180 group
f= p<0.05 vs. 1*30 group
g= p<0.05 vs. 1*60 group
h= p<0.05 vs. 5*30 group
i= p<0.05 vs. 5*60 group
j= p<0.05 vs. 10*30 group
k= p<0.05 vs. 10*60 group

Table 4.7: Effect of *in vitro* nicotine administration (at different concentrations: 0.1, 1, 5, 10 mM) on percentage of acrosome reacted human spermatozoa at increasing time points after exposure (30, 60, 120, 180 min).

		Acrosome Reacted Cells (%)			
		Timepoints (Min)			
		30	**60**	**120**	**180**
Nicotine (mM)	**C**	53.89046± 3.959339	52.39318± 3.959339	68.28274± 3.959339^{ab}	72.60488± 3.959339^{ab}
	0.1	51.28017± 3.959339	54.85325± 3.959339	62.51880± 3.959339^{c}	65.95232± 3.959339^{cd}
	1	40.71463± 3.959339^{ac}	56.49360± 3.959339^{f}	65.01059± 3.959339^{f}	69.61596± 3.959339^{fg}
	5	50.43880± 3.959339^{f}	59.22364± 3.959339	66.26950± 3.959339^{h}	71.80990± 3.959339^{hi}
	10	58.30764± 3.959339^{f}	64.57703± 3.959339^{bd}	70.82364± 3.959339^{ej}	73.29175± 3.959339^{j}

n=12.   a= $p<0.05$ vs. C*30 group
b= $p<0.05$ vs. C*60 group
c= $p<0.05$ vs. 0.1*30 group
d= $p<0.05$ vs. 0.1*60 group
e= $p<0.05$ vs. 0.1*120 group
f= $p<0.05$ vs. 1*30 group
g= $p<0.05$ vs. 1*60 group
h= $p<0.05$ vs. 5*30 group
i= $p<0.05$ vs. 5*60 group
j= $p<0.05$ vs. 10*30 group

4.2.3. Model 2(b): *In Vitro* Effect of Direct Nicotine Exposure on the Spermatozoal Parameters of Normal and Obese Wistar Rats

4.2.3.1. Total Motility

Results indicated that there were no indicative trends pertaining to increasing concentrations of nicotine (at pooled time points) and/or the influence of obesity on percentage total motile rat spermatozoa. There were also no significant differences between the control and treatment groups and values were largely comparable as seen in Figure 4.12.

	C	0.1	1	5	10
■ Obese	79.25	78.02	75.345	78.92	78.175
■ Non-Obese	80.91667	77.59167	76.85833	76.84167	76.27083

Figure 4.12: Effect of *in vitro* nicotine administration (at different concentrations: 0.1, 1, 5, 10 mM and pooled time points) on total motility of obese and non-obese rat spermatozoal total motility (n=6).

Results indicated that the total motility of non-obese Wistar rat spermatozoa decreased significantly as time periods after exposure increased (30> 120, 180at pooled nicotine concentrations, (30*non-obese vs. 120*non-obese: 81.333±4.089 % vs. 75.603±4.089 %; p<0.05; 30*non-obese vs. 180*non-obese: 81.333±4.089 % vs. 74.710±4.089 %; p<0.05). The effect of obesity did not affect total motility and results were relatively comparable for obese and non-obese groups as seen in Figure 4.13.

	30	60	120	180
Obese	77.12	79.064	77.948	77.636
Non-Obese	81.33333	79.13667	75.60333	74.71

n=6.   a= p<0.05 vs. C*30 group
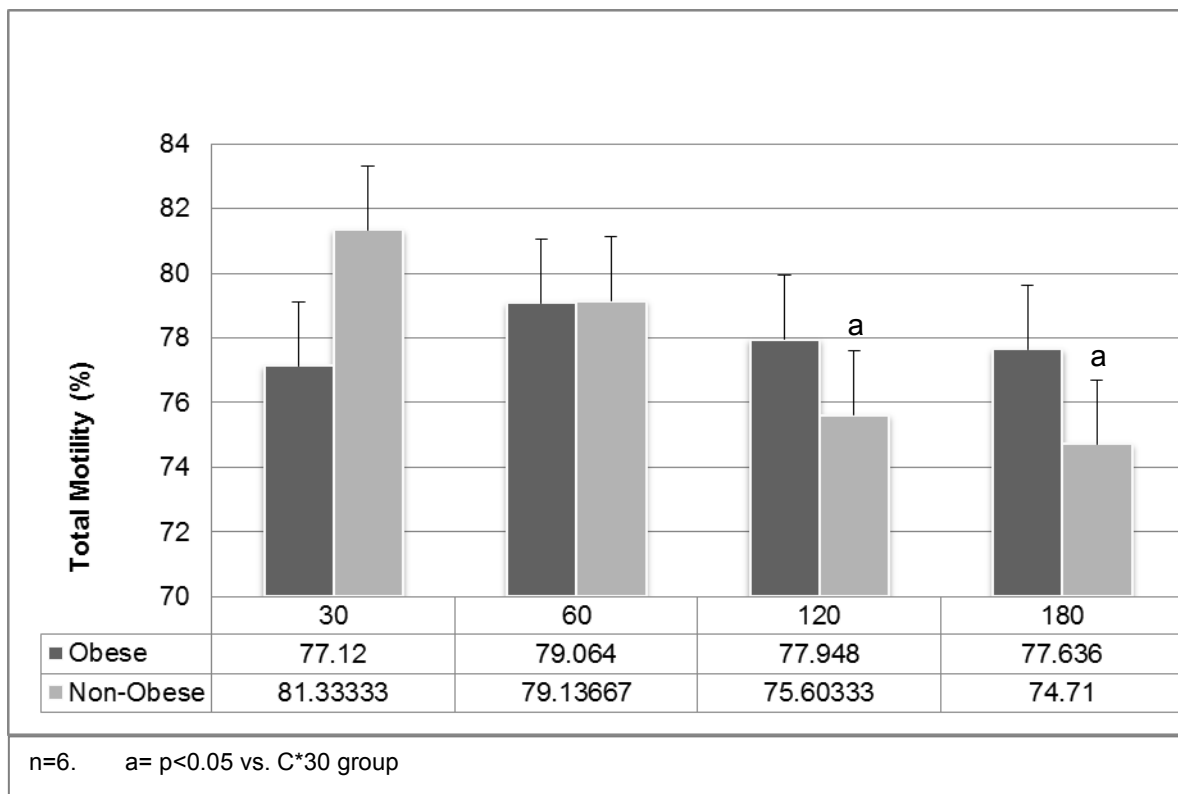
Figure 4.13: Effect of increasing timepoints after exposure (30, 60, 120, 180 min and pooled nicotine concentrations) on total motility of obese and non-obese rat spermatozoa (n=6).

4.2.3.2. Progressive Motility

Results indicated that the progressive motility of Wistar rat spermatozoa decreased significantly as nicotine concentrations increased (C, 0.1, 1, 5, 10) (at pooled time points) and was significantly decreased by obesity (non-obese>obese; C>0.1>1>5>10mM). The progressive motility C*obese, 1*non-obese, 5*non-obese and 10*non-obese groups were all significantly lower than that of the C*non-obese group (C*non-obese group vs. C*obese: 8.433±1.216 % vs. 3.315±1.333 %; $p<0.05$; C*non-obese group vs. 1*non-obese: 8.433±1.216 % vs. 5.838±1.216 %; $p<0.05$; C*non-obese group vs. 5*non-obese: 8.433±1.216 % vs. 5.350±1.216 %; $p<0.05$; C*non-obese group vs. 10*non-obese: 8.433±1.216 % vs. 3.704±1.216 %; $p<0.05$). The progressive motility of the 0.1*obese and 10*non-obese groups were all significantly lower than that of the 0.1*non-obese group (0.1*non-obese group vs. 0.1*obese: 7.038±1.216 % vs. 3.320±1.333 %; $p<0.05$; 0.1*non-obese group vs. 10*non-obese: 7.038±1.216 % vs. 3.704±1.216 %; $p<0.05$) as seen in Figure 4.14.
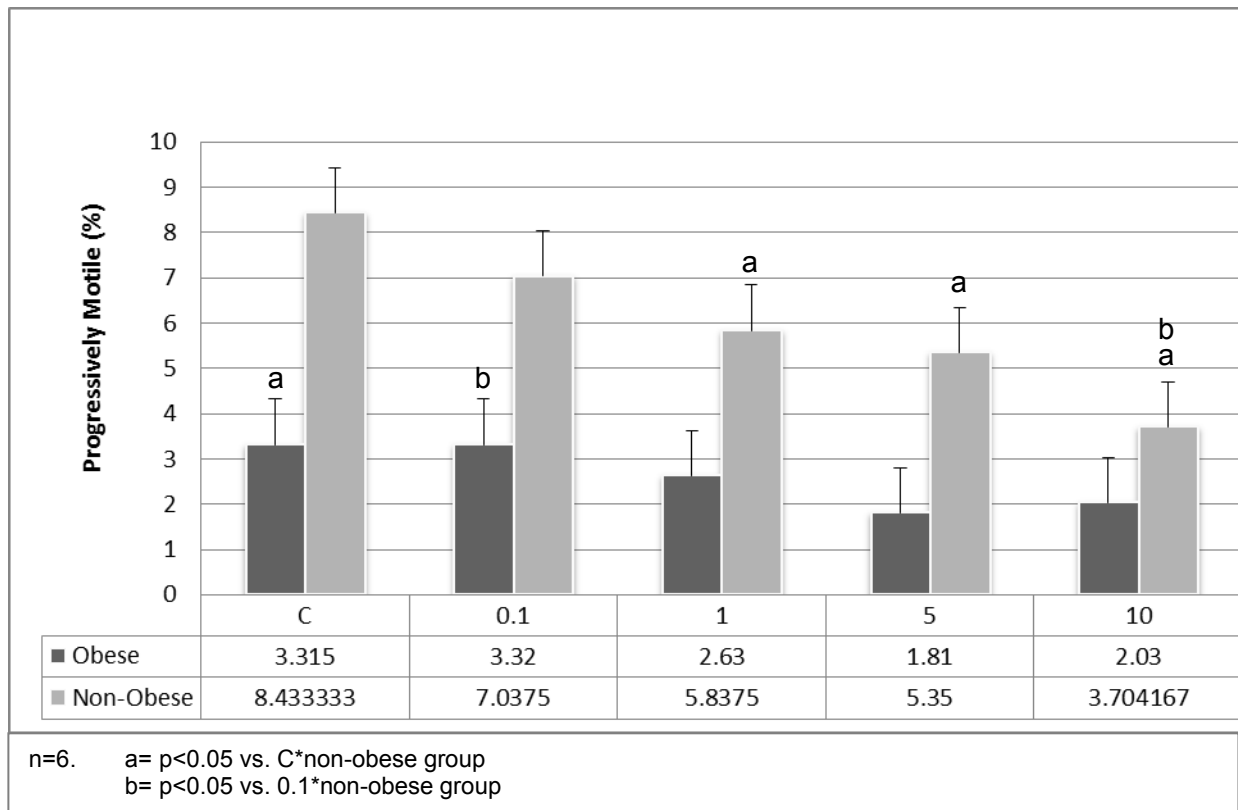
Figure 4.14: Effect of *in vitro* nicotine administration (at different concentrations: 0.1, 1, 5, 10 mM and pooled time points) on progressive motility of obese and non-obese rat spermatozoa (n=6).

Results indicated that the progressive motility of Wistar rat spermatozoa decreased significantly as time periods after exposure increased (30, 60, 120, 180) (at pooled nicotine concentrations) and was significantly decreased by obesity (non-obese>obese; 30, 60, 120, 180min). The progressive motility of the 30*obese, 120*non-obese and 180*non-obese groups were all significantly lower than that of the 30*non-obese group (30*non-obese group vs. 30*obese: 7.753±1.207 % vs. 2.736±1.322 %; $p<0.05$; 30*non-obese group vs. 120*non-obese: 7.753±1.207 % vs. 5.307±1.207 %; $p<0.05$; 30*non-obese group vs. 180*non-obese: 7.753±1.207 % vs. 3.793±1.207 %; $p<0.05$). The progressive motility of the 60*obese and 180*non-obese groups were significantly lower than that of the 60*non-obese group

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(60*non-obese vs. 60*obese: 7.437±1.207 % vs. 3.032±1.322 %; $p < 0.05$; 60*non-obese vs. 180*non-obese: 7.437±1.207 % vs. 3.793±1.207 %; $p < 0.05$) as seen in Figure 4.15.

	30	60	120	180
■ Obese	2.736	3.032	2.48	2.236
■ Non-Obese	7.753333	7.436667	5.306667	3.793333

n=6. a= p<0.05 vs. 30*non-obese group
b= p<0.05 vs. 60*non-obese group

Figure 4.15: Effect of increasing time points after exposure (30, 60, 120, 180 min and pooled nicotine concentrations) on progressive motility of obese and non-obese rat spermatozoa (n=6).

4.2.3.3. Viability

Results indicated that the viability of Wistar rat spermatozoa decreased significantly as nicotine concentrations increased (C, 0.1, 1, 5, 10) (at pooled time points) and was significantly decreased by obesity (non-obese>obese; C>0.1>1>5>10mM) as seen in Figure 4.16. The viability of C*obese, 1*non-obese, 5*non-obese and 10*non-obese groups were all significantly lower than that of the C*non-obese group (C*non-obese group vs. C*obese: 81.500±1.474 % vs. 63.000±1.614 %; $p < 0.05$; C*non-obese group vs. 1*non-obese: 81.500±1.474 % vs. 73.250±1.474 %; $p < 0.05$; C*non-obese group vs. 5*non-obese: 81.500±1.474 % vs. 67.208±1.474 %; $p < 0.05$; C*non-obese group vs. 10*non-obese:

81.500±1.474 % vs. 63.375±1.474 %; $p < 0.05$). The viability of the 1*obese, 5*obese and 10*obese groups were significantly lower than that of the C*obese group (C*obese vs. 1*obese: 63.000±1.614 % vs. 57.350±1.614 %; $p < 0.05$; C*obese vs. 5*obese: 63.000±1.614 % vs. 55.600±1.614 %; $p < 0.05$; C*obese vs. 10*obese: 63.000±1.614 % vs. 54.400±1.614 %; $p < 0.05$). The viability of the 0.1*obese, 5*non-obese and 10*non-obese groups were all significantly lower than that of the 0.1*non-obese group (0.1*non-obese group vs. 0.1*obese: 77.417±1.474 % vs. 63.350±1.614 %; $p < 0.05$; 0.1*non-obese group vs. 5*non-obese: 77.417±1.474 % vs. 67.208±1.474 %; $p < 0.05$; 0.1*non-obese group vs. 10*non-obese: 77.417±1.474 % vs. 63.375±1.474 %; $p < 0.05$). The viability of the 1*obese, 5*obese and 10*obese groups were all significantly less than that of the 0.1*obese group (0.1*obese vs. 1*obese: 63.350±1.614 % vs. 57.350±1.614 %; $p < 0.05$; 0.1*obese vs. 5*obese: 63.350±1.614 % vs. 55.600±1.614 %; $p < 0.05$; 0.1*obese vs. 10*obese: 63.350±1.614 % vs. 54.400±1.614 %; $p < 0.05$). The 1*obese, 5*non-obese and 10*non-obese group viability also proved to be significantly lower than the viability of the 1*non-obese group (1*non-obese vs. 1*obese: 73.250±1.474 % vs. 57.350±1.614 %; $p < 0.05$; 1*non-obese vs. 5*non-obese: 73.250±1.474 % vs. 67.208±1.474 %; $p < 0.05$; 1*non-obese vs. 10*non-obese: 73.250±1.474 % vs. 63.375±1.474 %; $p < 0.05$). The viability of the 5*obese and 10*obese groups presented significantly lower than that of the 5*non-obese and 10*non-obese groups respectively (5*obese vs. 5*non-obese: 55.600±1.614 % vs. 67.208±1.474 %; $p < 0.05$; 10*obese vs. 10*non-obese: 54.400±1.614 % vs. 63.375±1.474 %; $p < 0.05$).
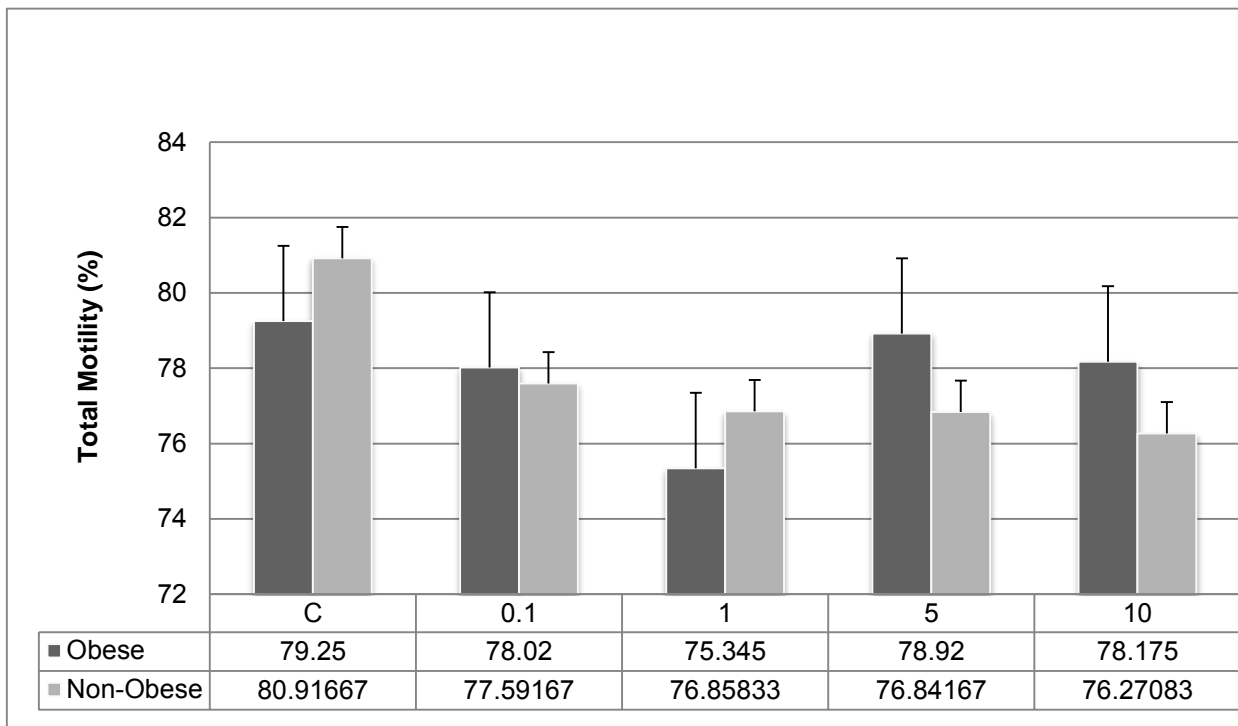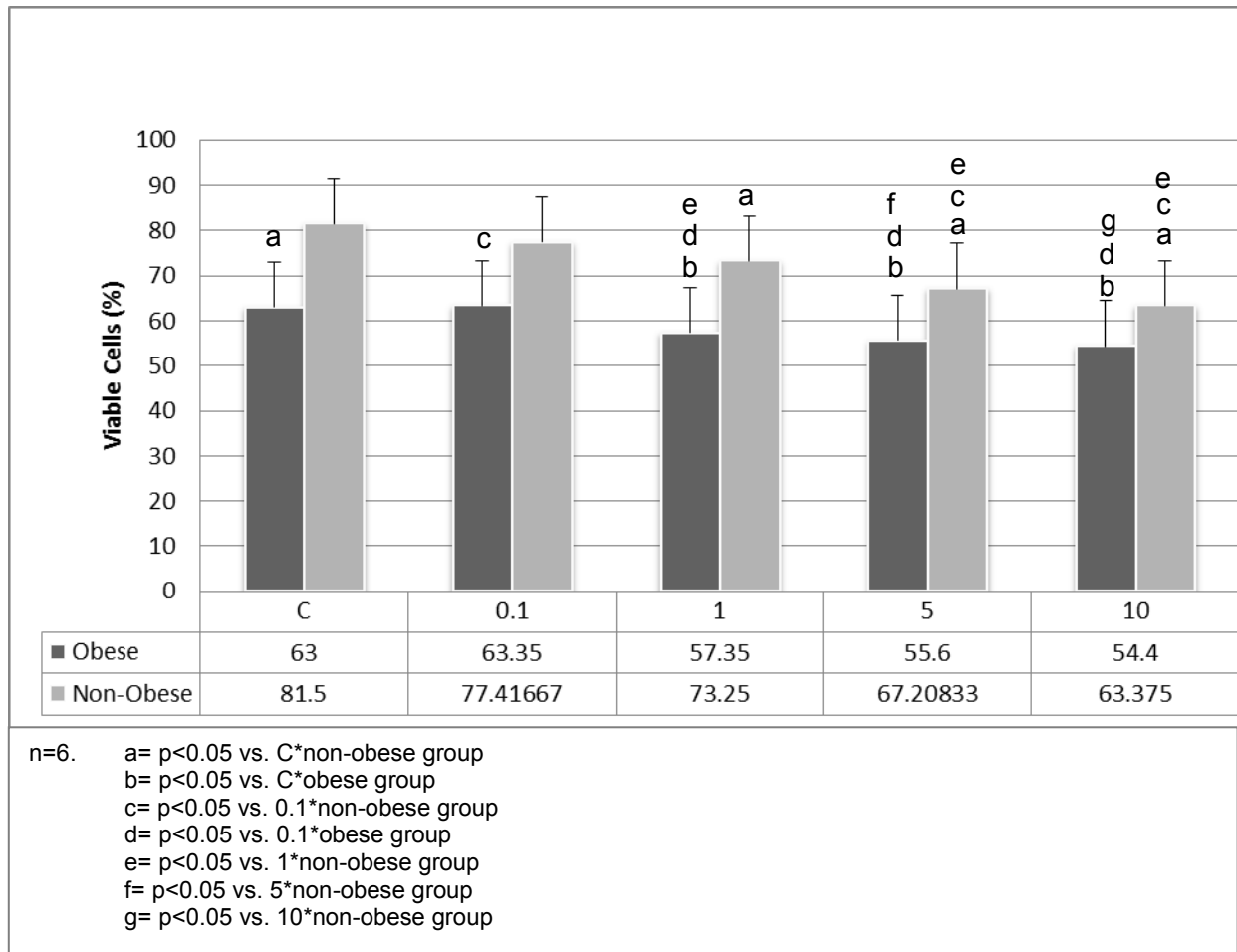
Figure 4.16: Effect of *in vitro* nicotine administration (at different concentrations: 0.1, 1, 5, 10 mM and pooled time points) on percentage viable obese and non-obese rat spermatozoa (n=6).

Results indicated that the viability of Wistar rat spermatozoa decreased significantly as time periods after exposure increased (30, 60, 120, 180, at pooled nicotine concentrations and was significantly decreased by obesity. (non-obese>obese; 30, 60, 120, 180min) as seen in Figure 4.17. The viability of the 30*obese, 120*non-obese and 180*non-obese groups were all significantly lower than that of the 30*non-obese group (30*non-obese group vs. 30 obese: 77.567±1.343 % vs. 68.640±1.472 %; $p<0.05$; 30*non-obese group vs. 120*non-obese: 77.567±1.343 % vs. 69.667±1.343 %; $p<0.05$; 30*non-obese group vs. 180*non-obese: 77.567±1.343 % vs. 66.433±1.343 %; $p<0.05$;). The viability of the 60*obese, 120*non-obese and 180*non-obese groups were all significantly lower than that of the

60*non-obese group (60*non-obese group vs. 60*obese: 76.533±1.343 % vs. 59.360±1.472 %; p<0.05; 60*non-obese group vs. 120*non-obese: 76.533±1.343 % vs. 69.667±1.343 %; p<0.05; 60*non-obese group vs. 180*non-obese: 76.533±1.343 % vs. 66.433±1.343 %; p<0.05). The viability of the 120*obese group was significantly lower than the 120*non-obese, 30*obese and 60*obese groups (120*obese vs. 30*obese: 54.120±1.472 % vs. 68.640±1.472 %; p<0.05; 120*obese vs. 60*obese: 54.120±1.472 % vs. 59.360±1.472 %; p<0.05; 120*obese vs. 120*non-obese: 54.120±1.472 % vs. 69.667±1.343 %; p<0.05). The viability of the 180*obese group was significantly lower than the 180*non-obese, 30*obese and 60*obese groups (180*obese vs. 30*obese: 52.840±1.472 % vs. 68.640±1.472 %; p<0.05; 180*obese vs. 60*obese: 52.840±1.472 % vs. 59.360±1.472 %; p<0.05; 180*obese vs. 180*non-obese: 52.840±1.472 % vs. 66.433±1.343 %; p<0.05). The 60*obese group's viability was significantly lower than the 30*obese group's viability (60*obese vs. 30*obese: 59.360±1.472 % vs. 68.640±1.472 %; p<0.05).

	30	60	120	180
■ Obese	68.64	59.36	54.12	52.84
■ Non-Obese	77.56667	76.53333	69.66667	66.43333

n=6. a= p<0.05 vs. 30*non-obese group
b= p<0.05 vs. 60*non-obese group
c= p<0.05 vs. 120*non-obese group
d= p<0.05 vs. 180*non-obese group
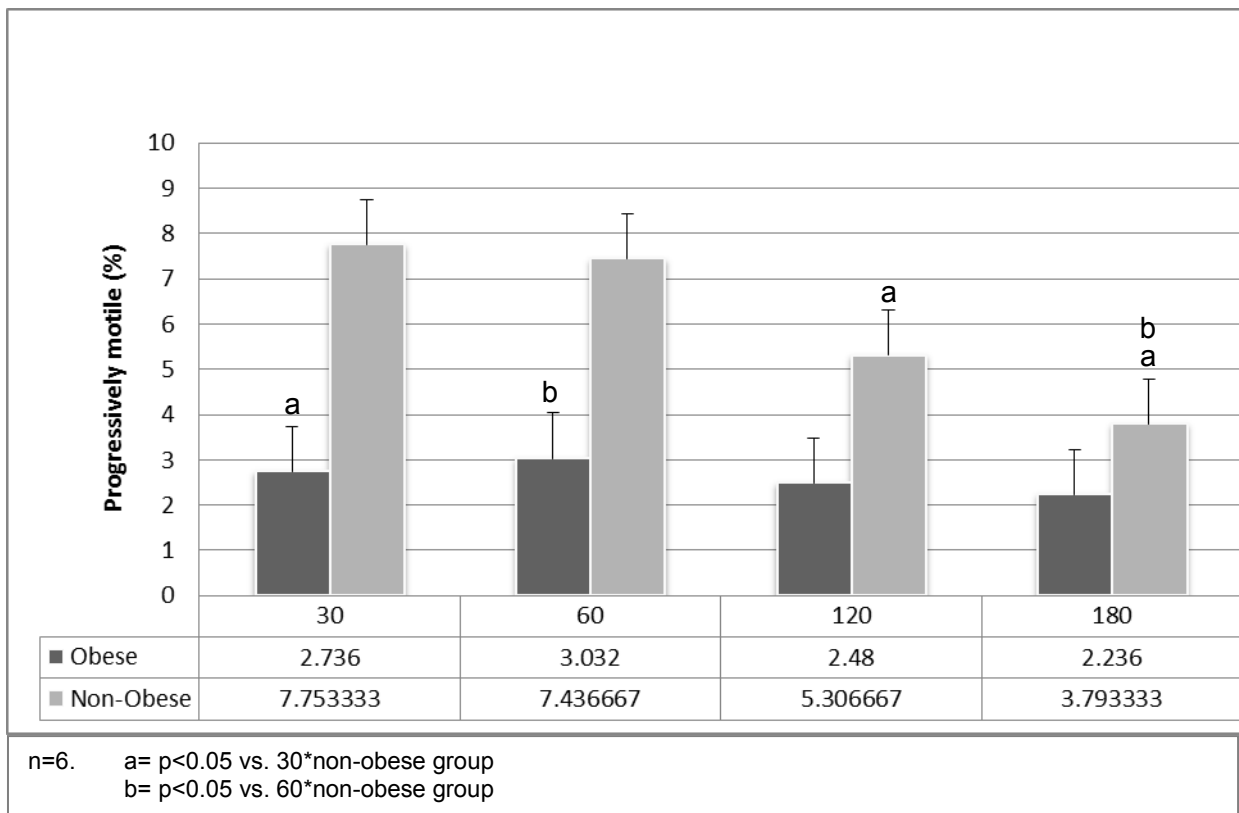e= p<0.05 vs. 30*obese group
f= p<0.05 vs. 60*obese group
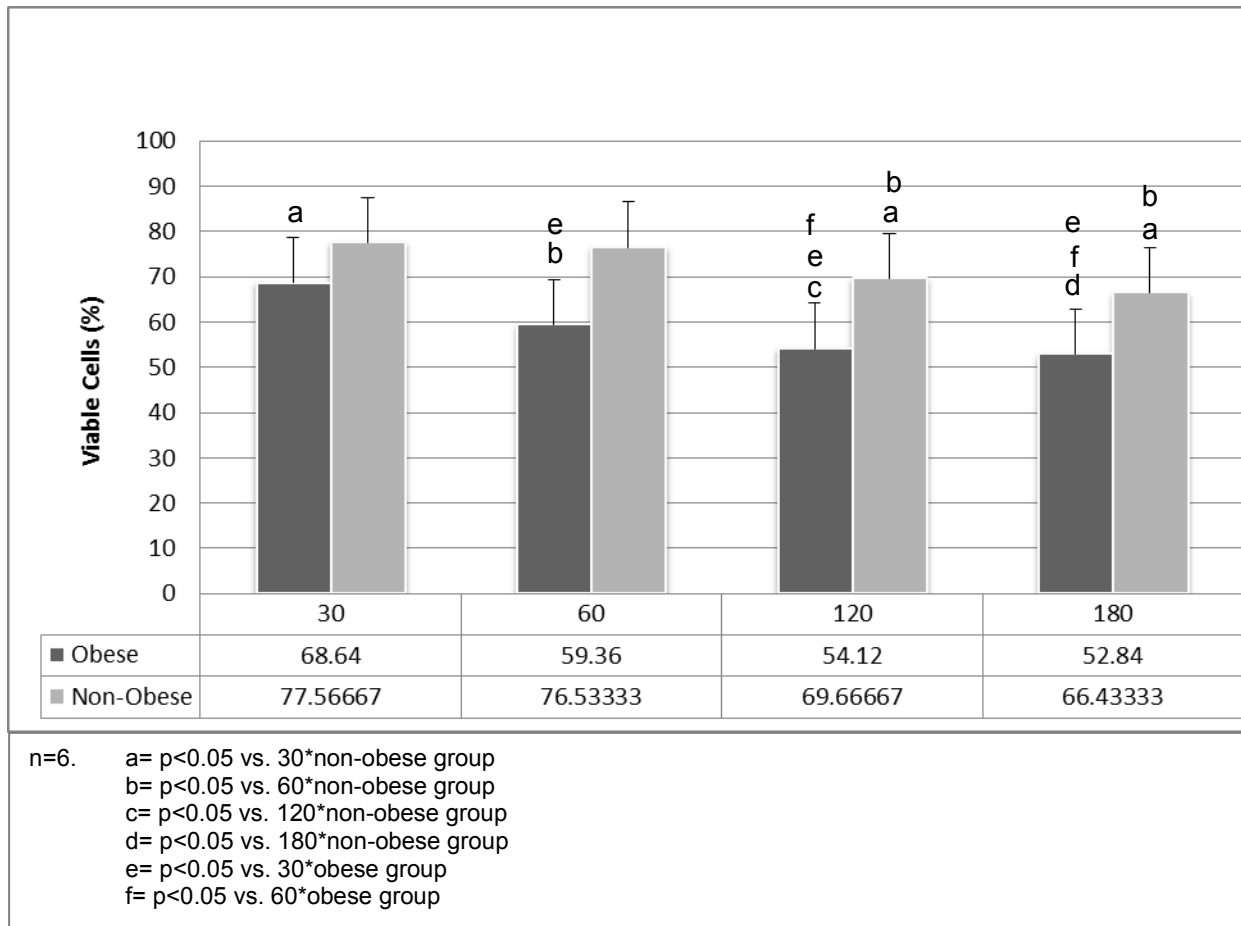
Figure 4.17: Effect of increasing time points after exposure (30, 60, 120, 180 min and pooled nicotine concentrations) on percentage viable obese and non-obese rat spermatozoa (n=6).

4.2.4. Rat spermatozoal parameter tables

The tables below (Tables 4.8-4.13) summarize all means of spermatozoal kinematic parameters (discussed in previous sections) of semen of obese and non-obese male Wistar rats (n=6) subjected to nicotine exposure at different concentrations (C, 0.1, 1, 5, 10 mM) measured at different timepoints (30, 60, 120, 180 min). For tables containing data of all parameters measured and relevant significance see Addendum A.

Table 4.8: Effect of *in vitro* nicotine administration (at different concentrations: 0.1, 1, 5, 10 mM and pooled time points) on total motility of obese and non-obese rat spermatozoa (n=6).

		Total Motility (%)			
Nicotine (mM)		**Obese**		**Non-Obese**	
	C	79.25000±4.522323		**C**	80.91667±4.128297
	0.1	78.02000±4.522323		**0.1**	77.59167±4.128297
	1	75.34500±4.522323		**1**	76.85833±4.128297
	5	78.92000±4.522323		**5**	76.84167±4.128297
	10	78.17500±4.522323		**10**	76.27083±4.128297

Table 4.9: Effect of increasing time points after exposure (30, 60, 120, 180 min and pooled nicotine concentrations) on total motility of obese and non-obese rat spermatozoa (n=6).

Total Motility (%)				
Timepoints (Min)				
	30	**60**	**120**	**180**
Obese	77.12000±4.479603	79.06400±4.479603	77.94800±4.479603	77.63600±4.479603
Non-Obese	81.33333±4.089299	79.13667±4.089299	75.60333±4.089299[a]	74.71000±4.089299[a]
n=6. a= p<0.05 vs. C*30 group				

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Table 4.10: Effect of *in vitro* nicotine administration (at different concentrations: 0.1, 1, 5, 10 mM and pooled time points) on progressive motility of obese and non-obese rat spermatozoa (n=6).

		Progressive Motility (%)					
Nicotine (mM)		**Obese**				**Non-Obese**	
	C	3.315000±	1.332583[a]		**C**	8.433333±	1.216476
	0.1	3.320000±	1.332583[b]		**0.1**	7.037500±	1.216476
	1	2.630000±	1.332583		**1**	5.837500±	1.216476[a]
	5	1.810000±	1.332583		**5**	5.350000±	1.216476[a]
	10	2.030000±	1.332583		**10**	3.704167±	1.216476[ab]

n=6.   a= $p<0.05$ vs. C*non-obese group
        b= $p<0.05$ vs. 0.1*non-obese group

Table 4.11: Effect of increasing time points after exposure (30, 60, 120, 180 min and pooled nicotine concentrations) on progressive motility of obese and non-obese rat spermatozoa (n=6).

Progressive Motility (%)			
Timepoints (Min)			
30	**60**	**120**	**180**
Obese 2.736000± 1.322208[a]	3.032000± 1.322208[b]	2.480000± 1.322208	2.236000± 1.322208
Non-Obese 7.753333± 1.207006	7.436667± 1.207006	5.306667± 1.207006[a]	3.793333± 1.207006[ab]

n=6.   a= $p<0.05$ vs. 30*non-obese group
        b= $p<0.05$ vs. 60*non-obese group

Table 4.12: Effect of *in vitro* nicotine administration (at different concentrations: 0.1, 1, 5, 10 mM and pooled time points) on percentage viable obese and non-obese rat spermatozoa (n=6).

Nicotine (mM)		Viable Cells (%)					
		Obese				**Non-Obese**	
	C	63.00000±	1.614223[a]		**C**	81.50000±	1.473578
	0.1	63.35000±	1.614223[c]		**0.1**	77.41667±	1.473578
	1	57.35000±	1.614223[bde]		**1**	73.25000±	1.473578[a]
	5	55.60000±	1.614223[bdf]		**5**	67.20833±	1.473578[ace]
	10	54.40000±	1.614223[bdg]		**10**	63.37500±	1.473578[ace]

n=6. a= p<0.05 vs. C*non-obese group
b= p<0.05 vs. C*obese group
c= p<0.05 vs. 0.1*non-obese group
d= p<0.05 vs. 0.1*obese group
e= p<0.05 vs. 1*non-obese group
f= p<0.05 vs. 5*non-obese group
g= p<0.05 vs. 10*non-obese group

Table 4.13: Effect of increasing time points after exposure (30, 60, 120, 180 min and pooled nicotine concentrations) on percentage viable obese and non-obese rat spermatozoa (n=6).

	Viable Cells (%)			
	Timepoints (Min)			
	30	**60**	**120**	**180**
Obese	68.64000± 1.471653[a]	59.36000± 1.471653[be]	54.12000± 1.471653[cef]	52.84000± 1.471653[def]
Non-Obese	77.56667± 1.343429	76.53333± 1.343429	69.66667± 1.343429[ab]	66.43333± 1.343429[ab]

n=6. a= p<0.05 vs. 30*non-obese group
b= p<0.05 vs. 60*non-obese group
c= p<0.05 vs. 120*non-obese group
d= p<0.05 vs. 180*non-obese group
e= p<0.05 vs. 30*obese group
f= p<0.05 vs. 60*obese group

Chapter 5: Discussion

This chapter is divided into two sections which is consistent with the study objectives. The first section describes the results obtained in the first segment of the study pertaining to *in utero* nicotine exposure and the second segment describes results as obtained in the *in vitro* nicotine exposure division of the study. At the end of each section a summary of the section's results may be found in addition to possible mechanisms of action relating to the observed results.

5.1 Model 1: Effect of *In Utero* Nicotine Exposure on Antioxidant Enzyme Activity and Lipid Peroxidation of the Reproductive System of Adult Male Wistar Rats

The objective of this section of the study was to evaluate the effect of *in utero* nicotine exposure on the antioxidant enzyme activity and LPO of the testicular tissue in adult male Wistar rats.

As previously noted, excess ROS production and associated OS have been shown to affect reproductive tissue. ROS generated by abnormal spermatozoa and leukocytes activates caspases that initiate a chain of downstream signalling reactions ultimately leading to DNA damage, by way of DNA methylation disruption, and apoptosis. The body's defence against this insult comes in the form of the primary antioxidant enzymes: SOD, CAT and GSH. By employing enzyme assays specific for these antioxidant enzymatic reactions, the antioxidant status of tissue can be monitored. When these enzymes are thus assayed for, in addition to other markers of tissue damage such as LPO, they can be utilized as indicative markers of the redox status of tissue.

5.1.1. Protein Concentration

Protein determination was done on all tissue homogenates in order to quantitatively express all biochemical parameter-units as a function of the amount of protein. Protein determination according to the BCA Protein Assay was achieved by the formation of a Cu^{2+}-protein

complex under alkaline conditions and the reduction of this complex to Cu^{1+} which was proportionate to the amount of protein present in the sample.

Results indicated that younger age groups displayed significantly higher protein concentrations than the older age groups (day 42>84>168) suggesting that as the testes develop, they expand in volume rather than weight and protein density and the lipid to protein ratio increases rapidly. This observation is of interest as, though the protein concentration of the testes decreased with age, primary antioxidant enzyme concentrations increased with age.

Protein concentrations of all treated groups displayed higher values than that of the control groups (N>C). This suggests that *in utero* nicotine exposure causes a significant increase in protein expression, but a decrease in antioxidant levels as the present study's other results reflected. Other studies have associated nicotine exposure to decreased testicular weights which could be attributed to a decrease in protein content.[18,151]

The N168 group was however, the only group significantly higher than its relevant control group suggesting that *in utero* nicotine exposure causes a significant increase in protein expression in later life. This observation seems to agree notably with a significant decrease between the day 168 old control and treatment groups in all three primary antioxidant enzymes reinforcing the idea that *in utero* nicotine exposure affects antioxidant status of testicular tissue in later life.

5.1.2. Superoxide Dismutase

SOD forms part of the primary antioxidant enzymatic system that protects the body against oxidative insult. Measurement of this parameter in the presence and absence of an exogenous factor, such as nicotine, will contribute to the understanding of the effect of said factor on the antioxidant profile of tissue. SOD activity was measured from the reaction of xanthine oxidase and hypoxanthine, in the presence of a tetrazolium salt, and the subsequent production of free radicals (O_2^-).

Results indicated that older age groups displayed significantly higher SOD concentrations than the younger age groups (day 42<84<168) suggesting that as testes develop with age antioxidant enzyme levels increase. This observation was uniform for all primary antioxidant enzymes. This agrees with a study done on brain and liver antioxidant enzyme levels which found a significant increase in all primary antioxidant enzymes of rats between 1 and 6 months of age.[151]

SOD concentrations of all treated groups displayed lower values than control groups (N<C). This observation was uniform for all primary antioxidant enzymes suggesting that *in utero* nicotine exposure decreases testicular levels of antioxidant enzymes. This observation is also in agreement with a study that reported a direct correlation between cigarette smoking and decreased SOD levels.[94]

The N168 group was however, the only group significantly lower than its relevant control group (N168 vs. C168: 45.653±0.644 Units/mg protein vs. 63.475±1.719 Units/mg protein) suggesting that *in utero* nicotine exposure causes a significant decrease in SOD expression in later life. This observation seems to remain notably uniform between the day 168 old control and treatment groups in all three primary antioxidant enzymes reinforcing the idea that *in utero* nicotine exposure affects antioxidant status of testicular tissue in later life.

5.1.3. Catalase

CAT also forms part of the primary antioxidant enzyme defence system of the body. CAT activity was measured by monitoring the peroxidatic function of CAT: CAT reacted with methanol in the presence of an optimal concentration of H_2O_2.

Results indicated that older age groups displayed significantly higher CAT concentrations than the younger age groups (day 42<84<168) suggesting that as testes develop with age antioxidant enzyme levels increase. This observation was uniform for all primary antioxidant enzymes. This agrees with a study done on brain and liver antioxidant enzyme levels which

found a significant increase in all primary antioxidant enzymes of rats between 1 and 6 months of age.[151]

The CAT concentrations of N42 and N84 groups displayed relatively similarly to that of the control groups (N=C) suggesting that *in utero* nicotine exposure does not affect CAT concentration at these two age groups. This observation is in agreement with a study that reported a correlation between cigarette smoking and slight decline in CAT levels, however not a significant correlation.[94]

The N168 group was however, the only group significantly lower than its relevant control group suggesting that *in utero* nicotine exposure causes a significant decrease in CAT expression in later life. This observation seems to remain notably uniform between the day 168 old control and treatment groups in all three primary antioxidant enzymes reinforcing the idea that *in utero* nicotine exposure affects antioxidant status of testicular tissue in later life. This decrease in CAT is also supported by an article that correlated cigarette smoke exposure to significant decreased CAT levels.[152]

5.1.4. Glutathione

GSH forms part of the primary antioxidant enzymatic system that protects the body against oxidative insult. Determination of the activity of GSH is based on the GSH mediated conversion of a luciferin derivative into luciferin in the presence of GST.

Results indicated that older age groups displayed higher, yet not significantly higher, GSH concentrations than the younger age groups (day 42<84<168) suggesting that as the testes develop with age, antioxidant enzyme levels also increase. This observation was uniform for all primary antioxidant enzymes. This agrees with a study done on brain and liver antioxidant enzyme levels which found an increase in all primary antioxidant enzymes of rats between 1 and 6 months of age.[151]

GSH concentrations of N42 and N84 groups was similar to their respective control groups (N=C) suggesting that *in utero* nicotine exposure does not affect GSH concentration of

offspring at these two age groups. This observation is in agreement with a study that did not reported any significant correlation between cigarette smoking and GSH levels.[152]

The N168 group was however, the only group significantly lower than its relevant control group suggesting that *in utero* nicotine exposure causes a significant decrease in GSH expression in later life. This observation seems to remain notably uniform between the day 168 old control and treatment groups with regards to all three primary antioxidant enzymes reinforcing the idea that *in utero* nicotine exposure affects antioxidant status of testicular tissue in later life.

5.1.5. Lipid Peroxidation

LPO is a well-established mechanism of antioxidant dysfunction and damage in cells. Lipid peroxides, such as MDA: a natural product of LPO, are unstable by-products of cellular OS. LPO was measured by monitoring the direct expression of a TBARS measurable product: MDA.

Results indicated that the d42 and d168 age groups displayed significantly higher MDA concentrations than the d84 group (day 42<84,168). This observation seems to suggest that rats with an age of 84 days old experience less OS and thus less membrane degradation, and subsequent LPO, than rats of 42 days and 168 days of age.

MDA concentrations of N84 and N168 were relatively comparable with the control groups (N=C) suggesting that *in utero* nicotine exposure does not affect MDA concentration at these two age groups.

The N42 group was however, the only group with MDA levels significantly lower than its relevant control group suggesting that *in utero* nicotine exposure causes a significant decrease in MDA expression in early life. This observation is in inconsistent with studies which have correlated cigarette smoke exposure with increased MDA expression and other markers of OS.[89,94,152]

5.1.6. Biochemical Parameter Summary and Possible Explanations for observed results

The table below (Table 5.1) summarizes the observed differences made by *in utero* nicotine exposure and increasing age on the antioxidant enzyme activity and LPO of Wistar rat testicular tissue.

Table 5.1: Summary of influence of age and *in utero* nicotine treatment on the antioxidant enzyme activity and LPO of rat testicular tissue.

Parameter	Age Influence	Control vs. Nicotine	Significant Group
Protein Concentration	day 42>84>168	N>C	d168
Superoxide Dismutase	day 42<84<168	N<C	d168
Catalase	day 42<84<168	N=C excl. d168 (N<C)	d168
Glutathione	day 42<84<168	N=C excl. d168 (N<C)	d168
Malondialdehyde	day 42<84,168	N=C excl. d42 (N<C)	d42

The present study observed a significant association of increasing age, decreasing protein concentration and increasing levels of antioxidant enzymes in testicular tissue as can be seen in the biochemical parameter summary results table: Table 5.1 and the parameter averages summary table and figures: Table 4.2 and Figure 4.1-4.2.

These results suggest that as the testes develop, they expand in volume rather than weight and protein density and the lipid to protein ratio increases rapidly. This was a unique and unexpected observation as the general expectation of protein concentration was to increase with age and development. This antioxidant enzyme increase agrees with a study done on brain and liver enzyme levels which found a significant increase in all primary antioxidant enzyme levels of rats between 1 and 6 months of age.[151] This observation is also in agreement with studies that concluded that antioxidant enzyme levels do not decline with aging, but are subject to several changes within the maturing rat testes.[152-153] This increase in antioxidant enzyme levels is probably a compensatory mechanism of the testicular tissue to cope with increasing exposure to oxidants due to aging and lifestyle exposure. Rats at the

age of 84 days old expressed less MDA and thus experienced less OS, membrane degradation and subsequent LPO than rats of 42 days and 168 days of age. This unexpected observation could be attributable to fluctuating levels of LPO during aging and/or the activity of the increasing levels of antioxidant enzymes.[151] A wide range of conflicting results concerning the effects of ageing on levels of LPO has been reported. These differences are attributed to differences in animal model, tissue monitored and assay type used.[154] In addition oxidatively damaged lipid is rapidly degraded and metabolized further complicating the analysis and interpretation of LPO results.[155]

The present study observed a significant association of *in utero* nicotine exposure, increased protein concentration and decreased levels of primary antioxidant enzymes (as seen in Figure 5.1). The increased protein concentration (as compared to controls) was an interesting observation as the authors expected nicotine exposure to decrease protein concentration in the testes as nicotine exposure has been related to testicular degradation. It has been long known that cigarette smokers have less body weight than non-smokers even though caloric intake is constant between the two.[117] The increase in protein concentration indicates, however, that the lipid: protein ratio in the testes of the nicotine exposed rats seems not to increase as rapidly as that of the control rats. This theory could be explained by the observation that cigarette smoking modulates metabolic pathways, via the modulation of metabolic hormones, resulting in less caloric storage.[156] Nicotine exposure also stimulates the autonomic nervous system causing the secretion of catecholamines and subsequently inhibiting the secretion of insulin and its anabolic functions.[157] Smoking has also been associated with less conversion of serum glucose and lipids to fat stores.[158-159] Thus free fatty acids in the bloodstream are plentiful in smokers. These fatty acids become an available source of energy for protein formation. This theory correlates with the results two other studies.[160-161] The observed enzymatic decreases are important observations and suggest that testicular primary antioxidant enzymes are susceptible to inactivation by *in utero* nicotine exposure. This observation is also in agreement with studies that reported a

direct correlation between cigarette smoking and decreased SOD, CAT and GSH levels.[94,152] Studies also reported a significant inverse correlation between SOD levels and leukocytospermia while leukocytospermia was positively correlated with cigarette smoke. Researchers thus attributed the decreased antioxidant enzyme levels to bone marrow activation due to cigarette smoking, leukocyte attraction and subsequent production of ROS. This theory is in concurrence with other studies that have correlated smoking, nicotine exposure and *in utero* nicotine exposure to OS and pre-apoptotic markers.[152,89,162,101] Studies have also linked nicotine exposure to decreases in circulating testosterone levels.[18] Testosterone is required to initiate and maintain spermatogenesis in the seminiferous tubules. Low serum testosterone levels have been associated with decreased weight and impaired function of the testis, epididymis and seminal vesicle. This reduction in testosterone is believed to originate from histological changes which have been reported in relation to nicotine exposure in the testes. Nicotine exposure is essentially toxic to the ultrastructure of the testes and causes degeneration of tissue and several cell types such as the epididymis, seminiferous tubules, germinal epithelium, spermatocytes, spermatids, Sertoli cells and Leydig cells causing the inhibition of the maturation of spermatozoa.[18,20,75,103,163] These findings are in accord with studies that have correlated nicotine exposure to decreased testicular weights.[18,150] Studies attribute these degradations to either vascular insufficiency-, direct cytotoxic effects on spermatogenic cells- or inhibition of prostaglandin synthesis-caused by nicotine exposure. The absence of testosterone and other reasons for disruption of reproductive maturation may thus decrease antioxidant enzyme levels of rats.[164] Of particular interest to the authors, was the observation that the treatment group of which each of the respective antioxidant enzyme levels were significantly less than the control group belonged to the 168 day old rats suggesting that *in utero* nicotine exposure affects antioxidant status of testicular tissue in later life. This observation is of particular note and supports the theory put forth by several articles that *in utero* nicotine exposure causes OS and affects male fertility in later life.[70,89] These results are confirmed and echoed by studies reporting an association between maternal smoking and decreased spermatozoal count,

volume and concentration in adulthood.[9-10;16] MDA concentrations were relatively comparable- and even slightly lower than the control groups were, suggesting that *in utero* nicotine exposure is associated with less markers of OS. This result could be due to the fact that, as previously mentioned, oxidatively damaged lipids are rapidly degraded and metabolized and thus difficult to quantify or due to the fact that TBARS tests are known to be non-specific.[165] Nevertheless, the N42 (pre-pubertal) group was observed to be the only group with MDA levels significantly lower than its relevant control group suggesting that *in utero* nicotine exposure caused a significant decrease in MDA expression in early life. This observation is inconsistent with studies which have correlated cigarette smoke exposure with increased MDA expression and other markers of OS.[89,94,152] The observation might however be explained by an intake of maternal antioxidants by the offspring (during infancy) via lactation decreasing levels of LPO. Studies have shown that among other substances, antioxidants and vitamin D are prominent micronutrients ingested during lactation.[166] This ingestion of antioxidants could result in a decrease in levels of OS in the rat offspring.

Rat Testicular Tissue

Maternal *(In Utero)* Nicotine Exposure

↑Protein Concentration

↓Antioxidant Enzymes

↓Lipid Peroxidation

Lipid: Protein ↓
Catecholamines ↑
Insulin ↓
Caloric Storage ↓
Free Fatty Acids ↑
Protein Formation ↑

Leukocytes ↑
Reactive Oxygen Species ↑
Oxidative Stress ↑
Testosterone ↓
Leydig Cell Function ↓
Sertoli Cell Function ↓

Lactational Antioxiants ↑
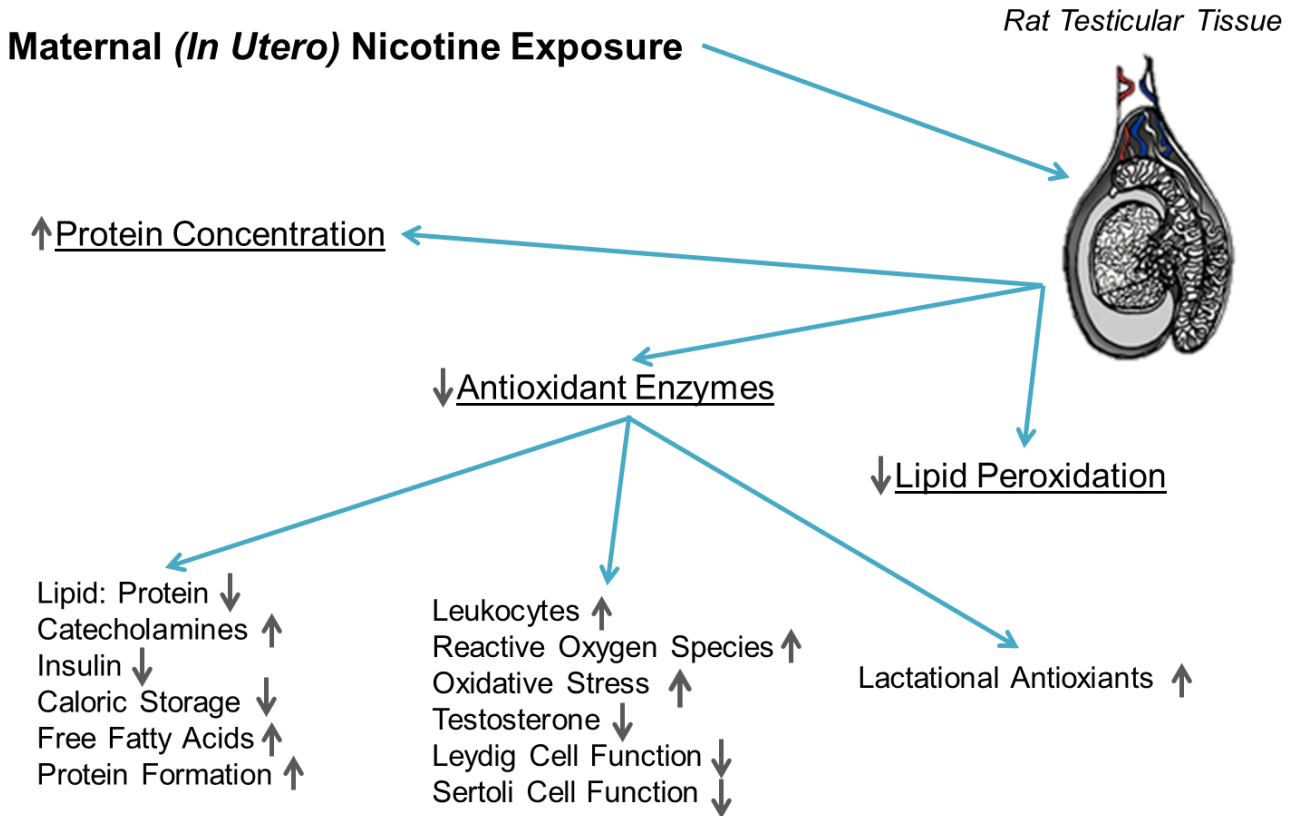
Figure 5.1: Effect of *in utero* nicotine exposure on testicular tissue of male Wistar rats and possible mechanisms of action for nicotine mediated effects.

5.2.   Model 2: *In Vitro* Effect of Direct Nicotine Exposure on the Spermatozoal Parameters of Human and Rat Subjects

The objective of this section was to evaluate the effects of high levels of *in vitro* nicotine exposure on the functional parameters of spermatozoa from human donors, normal rats and obese rats.

As previously discussed, several characteristics are of vital importance to spermatozoal function, when exposed to the environment of the cervical mucus and vagina, while attempting to reach and fuse with the egg. The total number (and concentration) of spermatozoa, their viability, motility and number of intact acrosomes are all crucial attributes that lead to the success of oocyte fertilization. By monitoring these parameters in the

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presence and absence of exogenous factors such as nicotine, the effect of said factor on the function of spermatozoa can be determined.

5.2.1. Model 2(a): *In Vitro* Effect of Direct Nicotine Exposure on the Spermatozoal Parameters of Human Subjects

5.2.1.1. Total Motility

Motility, as one of the primary spermatozoal functional parameters, plays an integral role in a spermatozoa's quest for fertilization. Spermatozoa have to be motile to reach the oocyte. There is a direct correlation between spermatozoal motility and pregnancy rates.[130,86] Total motility (fast progressive, slow progressive, non-progressive) was assessed by CASA as prescribed by the WHO.[130]

Results indicated that there were no indicative trends pertaining to increasing concentrations of nicotine or increasing time periods of measurement after nicotine exposure and influence on the percentage of motile human spermatozoa, except for significant reductions at the 120 min time point. This observation is in accordance with studies (*in vitro* and *in vivo*) that found that nicotine exposure and smoke exposure did not have a significant effect on spermatozoal motility.[24,115]

The 120min (after exposure) group did, however, show a significant relation between increasing concentrations of nicotine and a decrease in percentage total motile human spermatozoa (C>1>5>10 [120]) indicating that spermatozoal motility is susceptible to high concentrations of nicotine after 2 hours of exposure. This observation is in accordance with an *in vitro study* that reported that very high levels of nicotine inhibited spermatozoal kinematic parameters at increasing time points.[74] This observation agrees with progressive motility values, found in the present study, that decreased at the 120min time point.

5.2.1.2. Progressive Motility

Progressive motility is an important sperm functional characteristic, as spermatozoa have to be effective in their movement reach the oocyte. Research suggests that progressively

motile spermatozoa have a greater chance of fertilizing the oocyte than less progressive spermatozoa.[130,135] Progressive motility was assessed by CASA as prescribed by the WHO.[130]

Results indicate that neither increasing concentrations of nicotine nor increasing time periods after nicotine exposure have an effect on human spermatozoal progressive motility, except for significant reductions at the 120 min time point. This observation agreed with studies that found that *in vitro* and *in vivo* nicotine exposure and smoke exposure, respectively, did not have a significant effect on spermatozoal progressive motility.[24,115]

The 120min (after exposure) group did, however, show a significant association between increasing concentrations of nicotine and decrease in percentage progressively motile human spermatozoa (C>1>5>10 [120]). This observation also agrees with the *in vitro* study that reported that very high levels of nicotine inhibited spermatozoal kinematic parameters at increasing time points.[74]

5.2.1.3.   Viability

Viability is important to spermatozoal populations as higher levels of viable cells have a better chance of achieving fertilization. There is a direct correlation between declined spermatozoal viability and male infertility.[130,136] The viability assessment technique is based on the principle that the plasma membranes of dead and dying cells are permeable to membrane-impermeant stains. Viability status of spermatozoa was assessed microscopically by means of a dye exclusion staining technique (Eosin/Nigrosin) as prescribed by the WHO.[130]

Results indicated that the viability of human spermatozoa decreased as nicotine concentrations and time after exposure increased (30> 60> 120>180min; C>0.1>1>5>10mM]) indicating viability of spermatozoa are susceptible to decrease with increasing time points after ejaculation and increasing concentrations of nicotine. These

results again agree with studies that reported nicotine exposure and increasing time points to decrease spermatozoal viabilty.[17,74]

5.2.1.4.   Acrosome Reaction

Fusion of the spermatozoa and oocyte can occur only when spermatozoa shed their acrosomal sheath and penetrate the zona pellucida.[130] Spermatozoa contain serine proteases in the head (acrosin) which cause zona pellucida digestion. Appropriate timing of the AR is a requirement for fertilization of the oocyte and exposure to chemicals capable of inducing the AR prematurely might be deleterious to the fertilizing capability.[86,135-136] Exogenous factors such as nicotine are suspected of causing premature AR. The assessment of the AR has been used for many years to test the *in vitro* functional capacity of spermatozoa and there is a direct correlation between impaired acrosome reaction and male infertility.[167] The acrosome reaction status was visualized with fluorescently labelled lectins as prescribed by the WHO.[130]

Results indicated that the percentage of acrosome reacted human spermatozoa increased as nicotine concentrations and time after exposure increased (30<60<120<180min; C<0.1<1<5<10mM]) indicating that the acrosomal status of spermatozoa are adversely affected by extended periods of time after ejaculation and increasing concentrations of nicotine. These results are in accordance with studies that reported increasing time points to decrease spermatozoal kinematic parameters and smoke exposure to decrease fecundability and spermatozoal fertilizing capacity.[70,74,80]

5.2.2.   Model 2(b): *In Vitro* Effect of Direct Nicotine Exposure on the Spermatozoal Parameters of Normal and Obese Wistar Rats

5.2.2.1.   Total Motility

Results showed no indicative trends pertaining to increasing concentrations of nicotine and percentage of motile rat spermatozoa, and the values of the control and treatment groups were largely comparable. Results did, however, indicate that the motility of non-obese Wistar

rat spermatozoa decreased significantly as time periods after exposure increased (30>60>120>180). This observation is in accordance with a study that correlated increasing time points to decreased motility and semen quality.[74] The effect of obesity did not, however, affect total motility and results were relatively comparable for obese and non-obese groups. These results are in agreement with studies that reported no significant effect of either nicotine exposure or obesity on spermatozoal motility.[24,168]

5.2.2.2.   Progressive Motility

Results indicated that the progressive motility of Wistar rat spermatozoa decreased significantly as nicotine concentrations increased and was significantly decreased by obesity (non-obese>obese; C>0.1>1>5>10mM). The decrease in progressive motility resulting from increasing nicotine concentrations is in accord with several studies that report smoking and nicotine associated inhibition of spermatozoal kinematic parameters.[17,19,94,101] The decrease in progressive motility resulting from obesity, as observed, agrees with several studies that report obesity related inhibition of spermatozoal kinematic parameters.[120-121,168-169] Progressive motility of Wistar rat spermatozoa decreased significantly as time periods after exposure increased (30>60>120>180). The observation that increasing time points decreased progressive motility is in agreement with another study that found a similar result.[74]

5.2.2.3.   Viability

Results indicated that the viability of Wistar rat spermatozoa decreased significantly as nicotine concentrations increased and was significantly decreased by obesity (non-obese>obese; C>0.1>1>5>10mM). The decrease in viability resulting from increasing nicotine concentrations, as observed, is in accord with studies that report smoking and nicotine associated inhibition of spermatozoal vitality.[17,115] The decrease in viability resulting from obesity, as observed, is in accord with a study that reported an obesity related decrease in chromatin intact spermatozoa thus reflecting that obesity is related to DNA damage and pre-apoptotic markers.[120] Results indicated that the viability of Wistar rat

spermatozoa decreased significantly as time periods after exposure increased (30>60>120>180min). The observation that associated increasing time points to decreased viability is in agreement with another study that found a similar result.[74]

5.2.3. *In Vitro* Spermatozoal Parameter Summary and Possible Explanations for Observed Results

Tables 5.2 and 5.3 below summarize the observed differences made by increasing concentrations of *in vitro* nicotine exposure, increasing time points after collection and (in the case of rats) presence of obesity on the spermatozoal functional parameters of humans/Wistar rats. The tables also note any specific groups/parameters that were observed differently to the general trend found in the results.

Table 5.2: Summary of influence of increasing time exposure after ejaculation and increasing concentrations of *in vitro* nicotine treatment on the spermatozoal functional parameters of humans.

Parameter	Influence of time on parameter levels	Influence of nicotine on parameter levels	Group of exception to trend
Motility	--------------------------	--------------------------	120 min
Progressive Motility	--------------------------	--------------------------	120 min
Viability	30>60>120>180	C>0.1>1>5>10	--------------------------
Acrosome reaction	30<60<120<180	C<0.1<1<5<10	--------------------------

Table 5.3: Summary of influence of increasing time exposure after collection, increasing concentrations of *in vitro* nicotine treatment and the presence of obesity on the spermatozoal functional parameters of Wistar rats.

Parameter	Influence of time on parameter levels	Influence of obesity on parameter levels	Influence of nicotine on parameter levels	Group of exception to trend
Total Motility	30>60>120>180	-------------------	----------------	-------------------
Progressive Motility	30>60>120>180	Non-obese> obese	C>0.1>1>5>10	-------------------
Viability	30>60>120>180	Non-obese> obese	C>0.1>1>5>10	-------------------

 The present study observed a significant association between increasing time points after exposure and decreased human viability- and acrosomal status- of spermatozoa and decreased rat motility, progressive motility and viability of spermatozoa. Increasing *in vitro* nicotine concentrations were associated with decreased human viability- and acrosomal status- of spermatozoa and decreased progressive motility and viability of rat spermatozoa. Obesity was also associated with decreases in progressive motility and viability of rat spermatozoa. In human subjects a notable and unique observation was found between *in vitro* nicotine exposure and decreased motility and progressive motility of spermatozoa at a single time point: 120 min after ejaculation.

These results indicate that increasing time periods after ejaculation do not have a significant effect on human spermatozoal motility or progressive motility, but do however result in an inhibitory effect on viability and premature acrosome reaction of spermatozoa. Increasing time points after exposure were observed to decrease motility, progressive motility and viability of rat spermatozoa. This observation indicates that the viability and acrosomal status of human spermatozoa and the motility, progressive motility and viability are susceptible to decrease in function after extended periods of time after exposure and agrees with other studies that found increasing time points to decrease spermatozoal kinematic parameters in a dose-dependent manner.[17,74]

Results indicate that *in vitro* exposure to increasing high levels of nicotine does not have a significant effect on human spermatozoal motility or progressive motility, but does however have an inhibitory effect on viability and an effect eliciting premature excitation of acrosome reaction of spermatozoa (as seen in Figure 5.2). *In vitro* nicotine exposure also did not affect total motility, but decreased progressive motility and viability of rat spermatozoa. The authors attribute the lack of inhibition of total motility and progressive motility of human spermatozoa and total motility of rat spermatozoa by increasing nicotine concentrations, to small sample sizes and believe that if the sample sizes were bigger significant differences would be observed. This observation indicates that the viability and acrosomal status of human

spermatozoa and progressive motility and viability of rat spermatozoa are susceptible to decrease in function mediated by increasing levels of *in vitro* nicotine exposure, and is in accordance with studies that found that nicotine exposure and smoke exposure to decrease semen quality.[18,24,115] The observation that *in vitro* nicotine administration alters viability is in agreement with the observed decrease in acrosomal status as the premature initiation of acrosomal exocytosis seems to be related to perturbation of the plasma membrane stability.[170] The observed decrease in viability might be related to the histological changes which have been reported in relation to nicotine exposure in the testes. Nicotine exposure is essentially toxic several tissues and several cell types including spermatocytes and spermatids and thus might adversely affect the viability of spermatozoa.[18,20,75,103,163] Smoking and smoking components, such as nicotine, have also been linked to ROS production, oxidative DNA damage, OS and subsequent apoptosis. Elevated levels of ROS and apoptosis could be a causative factor in the reduction of viable cells as elevated levels of ROS and elevated levels of DNA damage have been found in the semen of smokers.[171-172] In addition to seminal OS, studies (including the present study) have also reported lower levels of antioxidant defence systems in the seminal plasma increasing spermatozoal susceptibility to OS.[171,173] Both of the theories discussed above (ROS and nicotine toxicity) may also be responsible for the reduction in motility and progressive motility which was observed in the 120min incubation group as premature initiation of acrosomal exocytosis seems to be related to perturbation of the plasma membrane stability.[170] The theory of the association of OS, apoptosis and poor motility is supported by a study that correlated high caspase levels, as markers of apoptosis, to poor spermatozoal motilty.[174]

In Vitro Nicotine Exposure (High Levels)

Spermatozoa

↑Acrosome Reacted Cells
(Human)

↓Viability *(Human & Rat)*

Nicotine Toxicity ↑
Spermatids/Spermatocytes ↓
Leukocytes ↑
Reactive Oxygen Species ↑
Oxidative Stress ↑
DNA damage, Apoptosis ↑
Antioxidants ↓

↓Progressive Motility
(Rat & Human [120min])

↓Total Motility
(Human [120min])

Caspase ↑
Membrane Stabiity ↓
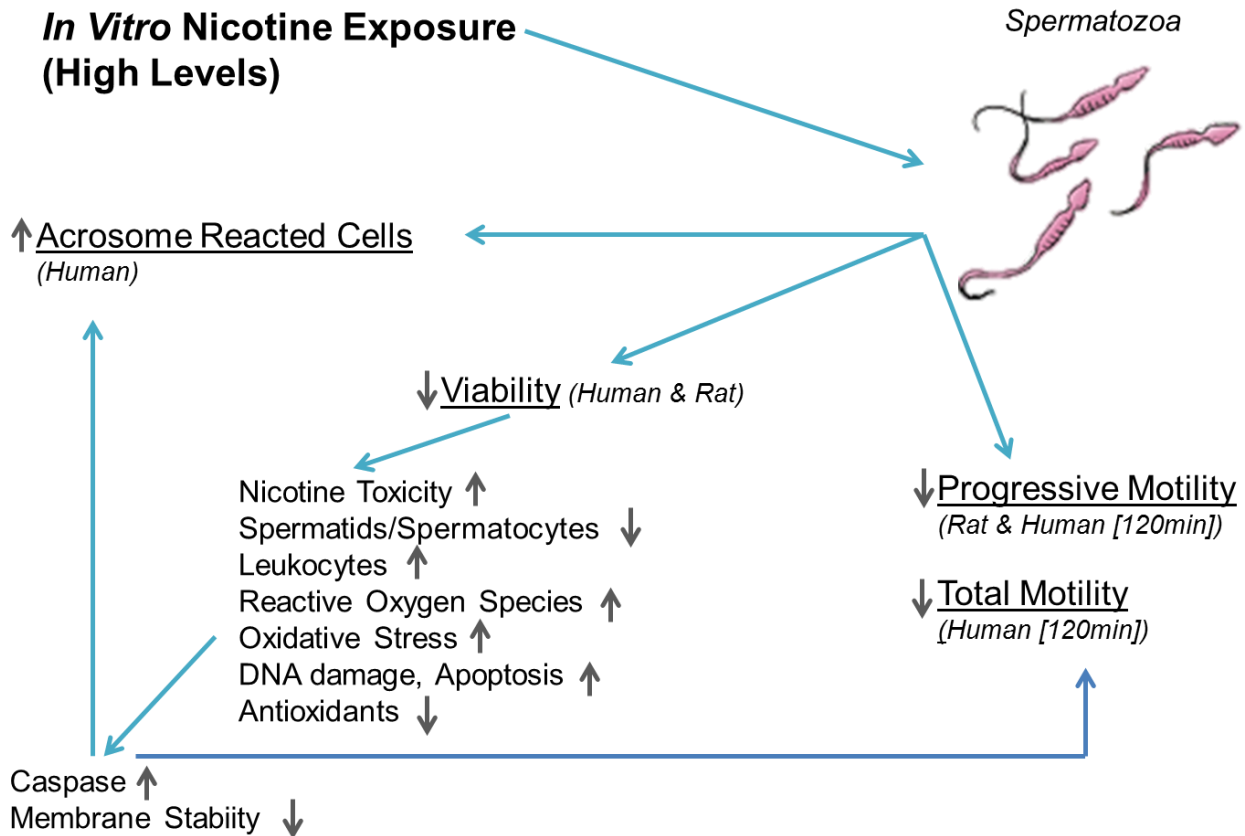
Figure 5.2: Effect of high levels of *in vitro* nicotine exposure on spermatozoa of male Wistar rats and humans and possible mechanisms of action for nicotine mediated effects.

Results indicated that obesity was observed to decrease progressive motility and viability of rat spermatozoa (as seen in Figure 5.3). These decreases were then further exacerbated by the *in vitro* exposure of nicotine to the spermatozoa. The decrease in viability resulting from obesity is in agreement with a study that reported an obesity related decrease in chromatin intact spermatozoa.[120] The decrease in progressive motility resulting from obesity agrees with several studies that report obesity related inhibition of spermatozoal kinematic parameters.[120-121,168-169] The authors attribute the lack of inhibition of total motility of spermatozoa by obesity to small sample sizes and believe that if the sample sizes were bigger significant differences would be observed. The decreases in progressive motility may be attributed to hormonal imbalances associated with obesity which adversely affect

spermatogenesis. Insulin resistance, sleep apnoea and increased negative feedback on gonadotropins lead to lower levels of sex hormone-binding globulin, higher levels of circulating oestrogens and subsequent decreased testosterone levels. The decrease in testosterone could affect the function of the seminiferous epithelium during maturation of spermatozoa and thus could lead to impairment of progressive motility.[118-121] The decrease in percentage of viable cells could be attributed to the induced states of systemic proinflammation caused by obesity. Systemic proinflammation is accompanied by increased activation of leukocytes, production of ROS and subsequent onset of OS and LPO. OS increases expression of apoptotic markers and also leads to DNA fragmentation. This occurrence may be responsible for the decline in viability.
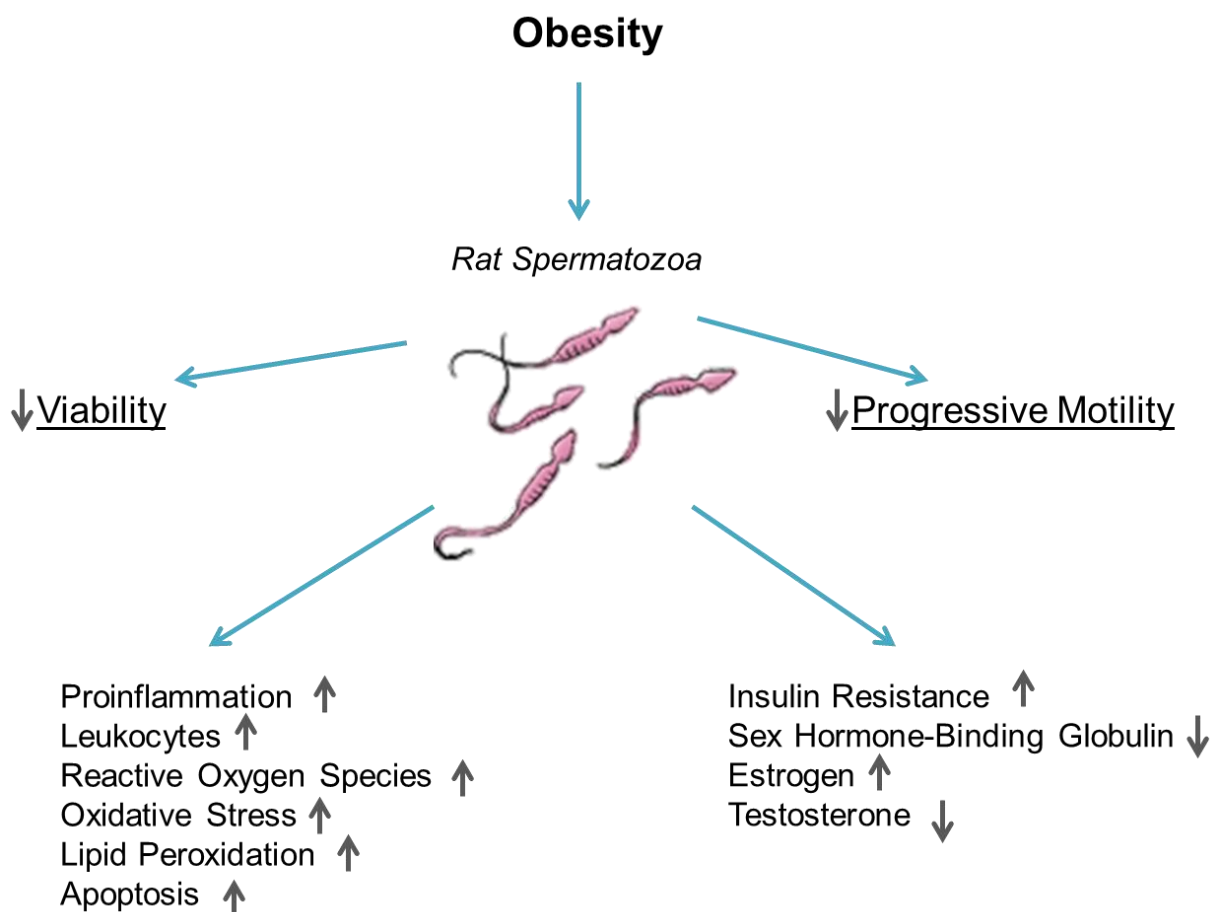
Figure 5.3: Effect of obesity on spermatozoa of male Wistar rats and possible mechanisms of action for nicotine mediated effects.

Chapter 6: Conclusion

The aim of the present study was to determine the effects of *in utero* and *in vitro* nicotine exposure on spermatozoal function and the antioxidant enzyme activity and lipid peroxidation of the male reproductive system.

Results indicate that *in utero* nicotine exposure was significantly associated with the following in testicular tissue:

- Increased protein concentration.

- Decreased levels of primary antioxidant enzymes.

- Decreased antioxidant status of testicular tissue in later life.

The observed increase in protein concentration is speculated to be related to the ability of smoking, and by association nicotine, to affect the protein: lipid ratio of testicular tissue. The observed enzymatic decreases are attributed to nicotine associated bone marrow activation, leukocyte attraction, excess production of ROS, induced OS and reduced activity of antioxidant enzymes. Of particular note was the observation that the treatment group, of which each of the respective antioxidant enzyme levels were significantly less than the control group, was the 168 day old rats suggesting that *in utero* nicotine exposure affects oxidative status of testicular tissue in later life.

In vitro administration of increasing high levels of nicotine was associated with the following:

- Decreased viability and increased acrosome excitation of human spermatozoa.

- Decreased motility and progressive motility of human spermatozoa, though only after 120min of incubation.

- Decreased progressive motility and viability of rat spermatozoa.

These adverse effects on the reproductive system can be attributed to nicotine associated toxicity of reproductive cells via perturbation of cellular plasma membrane stability. The nicotine concentrations that were used in the study are however much higher (50x-5000x)

than would ever be found in the seminal plasma of habitual smokers. The authors thus conclude that the adverse effects of smoking on the reproductive system cannot be accredited to the acute direct exposure of nicotine to spermatozoa in the seminal plasma.

Obesity was associated with the following in testicular tissue:

- Decrease progressive motility and viability of rat spermatozoa.
- Exacerbation of adverse effects of obesity on reproductive system by *in vitro* exposure of nicotine to spermatozoa.

The decrease in progressive motility may be attributed to hormonal imbalances associated with obesity which adversely affect spermatogenesis. The decrease in percentage of viable cells could be attributed to the induced state of systemic proinflammation caused by obesity. Systemic proinflammation is accompanied by increased activation of leukocytes, production of ROS, onset of OS, LPO and membrane degradation.

These results indicate that the acute *in vitro* exposure of spermatozoa to high levels of nicotine could adversely affect semen quality, but is probably not the causative factor in cigarette smoke responsible for the observed impairment of male fertility. *In utero* results reveal exposure of testicular tissue to maternal nicotine exposure adversely affects male fertility in later life and seems to elicit more detrimental effects on the reproductive system than that of direct exposure of nicotine to spermatozoa. Obesity also inhibits parameters of male fertility and these effects are exacerbated by smoking.

The authors thus conclude that lifestyle factors such as smoking, associated nicotine exposure and obesity may be additive factors to the impediment of male fertility and should be assessed and addressed by health care practitioners when diagnosing and treating infertility patients.

Future research is required incorporating the following:

- Larger sample sizes.

- *In vivo* exposure of nicotine to adult males (direct exposure model) measuring biochemical parameters and sperm function.

- Maternal nicotine exposure and sperm parameters.

- Advanced sperm parameter techniques (*in vivo* and *in vitro* exposure): seminal antioxidant and antioxidant enzyme measurement, intracellular and extracellular ROS production, morphology, DNA fragmentation.

- Difference between the acute vs. chronic effects of nicotine on reproductive system.

- The effects of cotinine on the reproductive system.

- Further studies on nicotine absorption and bioavailability within tissues.

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http://www.bmglabtech.com/images/apps/218-1.gif

178.   Sino Biological Inc. Western Blot Encyclopaedia Website. Available:

http://www.western-blot.us/procedure-of-western-blot/western-blot-sample-

preparation

Addendum A (Electronic): Complete Record of the Statistics of the Effect of Increasing Concentrations of *In Vitro* Nicotine Exposure, Increasing Time Points after Collection and (in the case of rats) Presence of Obesity on the Spermatozoal Functional Parameters of Humans/Wistar Rats

Files on disk:

A: (Word Doc) Tables containing standard error means and significant differences of the effects of increasing concentrations of *in vitro* nicotine exposure and increasing time points, after collection, on the spermatozoal functional parameters of humans.

B: (Word Doc) Tables containing standard error means and significant differences of the effects of increasing time points, after collection, and presence of obesity on the spermatozoal functional parameters of Wistar rats.

C: (Word Doc) Tables containing standard error means and significant differences of the effects of increasing concentrations of *in vitro* nicotine exposure and presence of obesity on the spermatozoal functional parameters of Wistar rats.

D: (Folder) Four files containing unsorted raw statistical data pertaining to project and all parameters measured.

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Addendum B: Publications Resulting from Study

ORIGINAL ARTICLE

In vitro effects of nicotine on human spermatozoa

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Summary

Washed human spermatozoa from 12 normozoospermic donors were treated with different concentrations of nicotine 0.1, 1.0, 5.0 and 10.0 mM and were compared to spermatozoa suspended in nutrient medium only (control). Computer-aided sperm analysis was used to assess sperm kinematic properties after 30, 60, 120 and 180 min of incubation. Viability was assessed by means of a dye exclusion staining technique (eosin/nigrosin), while acrosome-reacted cells were identified under a fluorescent microscope using fluorescein isothiocyanate–Pisum sativum agglutinin as a probe. Nicotine significantly reduced total motility, progressive motility, curvilinear velocity, amplitude of lateral head displacement, beat cross-frequency, viability and caused spontaneous acrosome reaction at concentrations of ≥5.0 mM after 2 and 3 h of exposure. Nicotine concentrations of 0.1 and 1.0 mM had no significant effect ($P < 0.05$) on spermatozoa except that 1.0 mM significantly decreased ($P < 0.05$) sperm progressive motility at 2 and 3 h of incubation as well as viability after 3 h of incubation. This study concludes that the occurrence of high levels of nicotine in the body and seminal fluid might adversely affect fertilisation capacity of human spermatozoa through a mechanism that involves decreased motility, viability and premature induction of the acrosome reaction.

Introduction

Cigarette smoking has become a very common occurrence and a serious health and economic issue for society. Tobacco usage, according to the World Health Organisation (WHO), is associated with approximately one-third of the world's population older than 15 years of age (Mohammed *et al.*, 2008). Nicotine is one of the main components of the particulate phase of tobacco combustion. It has been found to be very harmful to the human body after its quick absorption through the respiratory tract, mouth mucosa and skin, while 80–90% is metabolised by the liver, kidney and lungs (Benowitz *et al.*, 2006). Nicotine and its metabolite (cotinine) have been found in serum, urine, saliva and milk, and more recently significant levels have been found in smokers' seminal plasma in subjects exposed to environmental tobacco smoke (Pacifici *et al.*, 1995).

Sperm motility, capacitation and acrosome reaction (AR) are prerequisites to achieve oocyte fertilisation. The AR is important during fertilisation because fusion of the spermatozoa and oocyte can only occur when spermatozoa shed their acrosomal sheath, penetrate the zona pellucida and fuse with the oocyte membrane (Yanagimachi, 1994). Spermatozoa contain serine proteases in the head (acrosin) that cause zona pellucida digestion (Harrison, 1983), enabling the spermatozoa to penetrate the oocyte (Saling & Storey, 1979). Only acrosome-intact spermatozoa can digest the zona pellucida. Appropriate timing of the AR is a requirement for fertilisation of the oocyte, and exposure to chemicals capable of inducing the AR prematurely might be deleterious to the fertilising capability (Cummins *et al.*, 1986). Various exogenous factors such as nicotine and ionising-radiation are believed to cause premature AR. The assessment of the AR has been used for many years to test the *in vitro* functional capacity of spermatozoa (Kumi-Diaka & Townsend, 2003; Suri, 2005).

Several researchers have documented the effects of nicotine on reproductive function. Nicotine has been reported to affect sperm motility *in vivo* in both habitual smokers (Saaranen *et al.*, 1987) and animal models (Oyeyipo *et al.*, 2010). *In vitro* studies available on the effect of nicotine on sperm motion characteristic remain few in number and controversial (Reddy *et al.*, 1995; Gandini *et al.*, 1997). In addition, nicotine has been shown to induce

adverse effects on fertility potentials in rats (Oyeyipo *et al.*, 2011). However, the mechanism by which nicotine reduces fertility potentials is still unknown.

The aim of this study was therefore to evaluate the *in vitro* effect of high levels of nicotine on sperm motility and kinematic parameters, viability and acrosome status in normozoospermic semen samples from nonsmokers at different nicotine dosages and durations of exposure to ascertain at what concentration nicotine inhibits sperm parameters.

Materials and methods

Chemicals

Nicotine, fluorescein isothiocyanate–Pisum sativum agglutinin (FITC-PSA), Hams F10 medium containing 3% bovine serum albumin (HAMS-BSA), phosphate-buffered saline (PBS), eosin and nigrosin were obtained from Sigma Chemicals (St Louis, MO, USA). The nicotine working solutions were freshly prepared every third day and stored at 4 °C in a dark glass container to prevent light exposure.

Preparations of sperm samples

Healthy donors of between 19 and 26 years of age ($n = 12$) were randomly recruited for this study, and they all provided informed consent for a research protocol that received IRB approval (Tygerberg, South Africa). Smoking was the only exclusion criteria for the study. Exposure of donors to environmental and second-hand smoke was not controlled for, but the level of nicotine in circulation resulting from environmental smoke exposure as reported by Pacifici *et al.* (1995) is of such a low level that it is not of consequence to such a study. Fresh semen samples were obtained by masturbation after 2–7 days of sexual abstinence displaying functional parameters of concentration between 12 and 16×10^6 ml^{-1}, volume between 1.4 and 1.7 ml and total count between 33 and 46×10^6 per ejaculate as specified by the WHO (1999) guidelines to be regarded as fertile. Samples were left to liquefy for 30 min (37 °C, 5% CO_2, 95% humidity) before processing. Motile sperm fractions were retrieved from the samples using a double wash ($400 \times g$, 5 min) swim-up technique in Hams F10 medium containing 3% bovine serum albumin. The supernatant containing motile spermatozoon was collected and divided into equal aliquots containing 5×10^6 spermatozoa ml^{-1}.

Samples were exposed to various concentrations of nicotine (0.1, 1, 5 and 10 mM) of much higher levels than that present in the seminal plasma of smokers to establish at what concentration nicotine adversely affects

sperm parameters. Nicotine concentrations of between 70 μg l^{-1} (0.00043 mM) and 300 μg l^{-1} (0.00185 mM) are commonly found in the semen of casual (1–10 cigarettes per day) and habitual smokers (>30 cigarettes per day; Pacifici *et al.*, 1993) but have rarely, without controversy, been reported to affect semen parameters at these concentrations (Goverde *et al.*, 1995; Gandini *et al.*, 1997; Cope, 1998; Povey *et al.*, 2012). Samples were incubated for different time periods at 37 °C, 5% CO_2 and 95% humidity. Sperm motion analysis was performed on 5-μl aliquots from each tube at 30, 60, 120 and 180 min respectively.

Motility parameters

Sperm motility parameters were measured by means of computer-aided sperm analysis (CASA) using the sperm class analyser® (SCA®; Microptic, Barcelona, Spain) after incubation and exposure to nicotine at different concentrations and different time points. The system analysed the total motility, progressive motility (percentage of A + B level of spermatozoa) and kinematic and velocity parameters such as curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), amplitude of lateral head displacement (ALH) and beat cross-frequency (BCF).
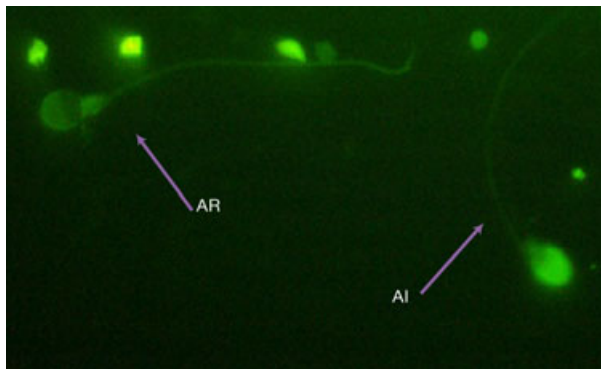
Acrosome reaction

Spermatozoa that were exposed to different concentrations of nicotine, as well as a control sample not exposed to nicotine, were left to incubate for 3 h to allow nicotine to induce premature AR. The extent of the AR was assessed by creating smears of samples on spotted slides, leaving them to air dry and then fixing them in cold ethanol (4 °C, 30 min; WHO, 1999). Spots were covered with FITC-PSA solution (125 μg ml^{-1}) and incubated for 30 min in a dark humidified atmosphere. Slides were subsequently rinsed with distilled water to eliminate excess probe, exposed to DACO Antifade (Glostrup, Denmark) mounting medium and observed under a fluorescence microscope for probe visualisation (Esteves *et al.*, 2007). At least 200 cells were evaluated per slide. Spermatozoa with bright green fluorescence at the acrosome region were counted as acrosome intact, while those with pale-green fluorescing regions were counted as acrosome-reacted spermatozoa as shown in Fig. 1.

Cell viability

Viability was assessed by means of a dye exclusion staining technique (Eosin/Nigrosin). Stained cells were identified and expressed as the percentage of nonviable cells

(Eliasson, 1977). Unstained (intact) and red stained (with damaged membrane) spermatozoa were counted at ×100 magnification.

Fig. 1 Photomicrograph illustrating acrosomal status of spermatozoa assessed by fluorescein isothiocyanate–Pisum sativum agglutinin (FITC-PSA). AI: Acrosome intact: bright green fluorescence in the acrosomal region. AR: Acrosome reacted: pale-green fluorescence in the acrosomal region.

Statistical analysis

All data were expressed as mean ± SEM. A paired *t*-test was also carried out between the control and different treatments at each observation time point for the various parameters. One-way analysis of variance (ANOVA) was used followed by Duncan's multiple range test for pairwise comparison. All statistical comparisons and tests were performed using Statistical Package for Social Sciences (SPSS Inc, Chicago, IL, USA) for windows. Statistical significance was set at $P < 0.05$.

Results

Motility

All kinematic parameters that were affected significantly by the different nicotine concentrations (0.1, 1, 5 and 10 mM) at various time points (30, 60, 120 and 180 min) are shown in Fig. 2. However, parameters were generally affected by the three higher concentrations of nicotine after 120 min of exposure. From the results, it is evident that total motil-
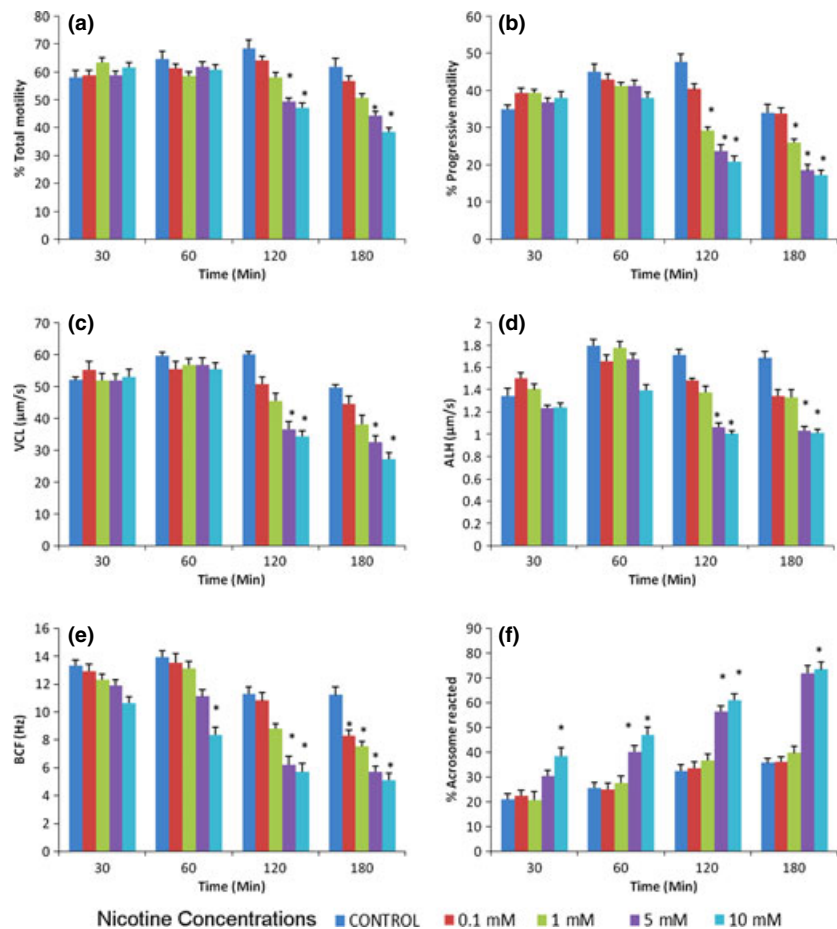
Fig. 2 Effect of different nicotine concentrations on sperm motion and kinematic parameters (a–e) as well as acromal status (f) after 30, 60, 120 and 180 min of nicotine exposure and incubation. Values are expressed as mean ± SEM of 12 samples *P < 0.05 versus control. VCL, curvilinear velocity; ALH, amplitude of lateral head displacement; BCF, beat cross-frequency.

ity was significantly reduced at concentrations of 5 and 10 mM after 120 min (49.2 ± 1.04% and 47.1 ± 1.47 versus 68.3 ± 2.42% respectively; $P < 0.05$) and 180 min of exposure (44.3 ± 2.64% and 38.3 ± 1.58 versus 68.3 ± 2.11% respectively; $P < 0.05$). VCL and ALH were affected similarly with the addition of significant reduction at a concentration of 1 mM. Concentrations of nicotine (1, 5 and 10 mM) affected progressive motility at the same time points (120 and 180 min; 29.2 ± 2.03%, 23.6 ± 2.28% and 20.7 ± 2.43% versus 47.6 ± 2.87% respectively; $P < 0.05$; 25.9 ± 2.83%, 18.4 ± 2.56% and 17.1 ± 2.12% versus 33.9 ± 2.75% respectively; $P < 0.05$), while VCL, ALH and BCF were significantly reduced ($P < 0.05$) at time point 120 min (5 and 10 mM).

Viability

Viability of spermatozoa was significantly decreased ($P < 0.05$) by nicotine at concentrations of 5 and 10 mM after 120 and 180 min of exposure. The 0.1 and 1.0 mM nicotine exposure groups had a comparable value with the control throughout the incubation period except for the viability at 1.0 mM of nicotine exposure that was significantly decreased after 3 h of incubation as shown in Table 1.

Acrosome reaction

There was a significant increase in acrosome-reacted spermatozoa after 30 min of exposure to 10 mM of nicotine. At 60, 120 and 180 min of exposure, both the 5 and 10 mM treatment groups caused a significant increase in acrosome-reacted spermatozoa (Fig. 2).

Table 1 The effect of nicotine exposure (at different concentrations: 0.1, 1.0, 5.0 and 10 mM) on sperm cell viability (at different time intervals: 30, 60, 120 and 180 min) using eosin/nigrosin dye exclusion stain

Dose	30 Min	60 Min	120 Min	180 Min
Control	75.5 ± 8.01[a]	69.1 ± 7.40[a]	64.4 ± 9.14[a]	57.5 ± 8.56[a]
0.1 mM	72.4 ± 9.94[a]	64.5 ± 8.50[a]	62.5 ± 9.11[a]	53.5 ± 10.10[a]
1.0 mM	68.8 ± 9.75[a]	63.3 ± 11.98[a]	57.1 ± 9.75[a]	40.0 ± 7.56[b]
5.0 mM	60.9 ± 7.32[a]	49.8 ± 8.47[a]	44.7 ± 8.33[b]	40.4 ± 7.33[b]
10 mM	58.6 ± 7.09[a]	45.9 ± 6.58[a]	42.8 ± 6.05[b]	36.6 ± 6.05[b]

Values are expressed as means ± SEM of 12 subjects. Means in columns not sharing common superscript letters are significantly different; $P < 0.05$.

Discussion

In this study, the effect of nicotine was assessed on several motility parameters: progressive motility, total motility, VCL, VSL, VAP, ALH, BCF as well as the viability and acrosome status of the spermatozoa. By assessing the effect of nicotine on washed spermatozoa, the authors attempted to establish whether nicotine, as a substance, affected healthy functional spermatozoa. It can only be speculated that the effect of nicotine exposure on an unwashed semen sample would be similar or even slightly exacerbated as the environment would contain higher levels of ROS (intracellular and extracellular). The present study shows that direct exposure of spermatozoa to the study's lowest concentration ranges of nicotine (0.1 and 1 mM) had no or very little effect on various functional parameters.

Study findings indicated that sperm motion characteristics and viability decreased after 120 min of incubation at high doses (5 and 10 mM). The data also suggest that nicotine reduces human spermatozoa kinematic parameters as evidenced by reduced total and progressive motility as well as VCL and ALH. The spermatozoa motility parameters with note to velocity are of significant predictive value to male fertility (Larsen et al., 2000). Studies have shown that VCL rather than VSL of spermatozoa can be correlated with improved rate of fertilisation (Liu et al., 2004), while ALH has previously been correlated with the efficacy of cervical mucus penetration (Aiken et al., 1986). Bongso et al. (1989) noted a higher ALH displayed by spermatozoa of fertile men than in those of an infertile group. Improved ALH may improve VCL by exaggerating spermatozoa head movement from side to side.

This study also suggests a significant decrease in the viability of spermatozoa at concentration ≥5.0 mM after 120 and 180 min of exposure, while 1.0 mM also significantly decreased viability after 180 min of incubation.

The reduction in the viability agrees with reduction in the progressive sperm motility and is in consonance with previous studies (Pacifici et al., 1993). This is deduced from observing dead spermatozoa taking up the nigrosin stain due to compromised cell membranes, serving as evidence that they are dead. This result may be seen due to the action of nicotine as a spermicide affecting maturing or matured spermatozoa.

The observed increase in the number of acrosome-reacted spermatozoa therefore suggests that the number of spermatozoa with intact acrosomes, able to interact with the zona pellucida, is reduced, suggesting a possible link to compromised fertilisation (Darszon et al., 2001). The premature excitation of this process by nicotine could thus decrease the chance that spermatozoa will fertilise the egg in the oviductal lumen.

The mechanism by which nicotine prematurely stimulates the AR was not investigated in this study, and further studies are recommended. It can be speculated, however, that this observation might be mediated through increased ROS production. Studies have, however, shown interesting links between decreased sperm activity, premature AR (Kato *et al.*, 2002) and reduced sperm motility as evidenced by increased total and progressive motility as well as VCL and ALH (Liu *et al.*, 2008). The premature initiation of acrosomal exocytosis seems to be related to perturbation of the plasma membrane stability. The mechanism whereby this happens is postulated not to be linked to the AR involving a premature activation of the receptor-mediated process, but rather reflecting an inherent fragility of the sperm membrane leading to a receptor independent acrosomal loss (Esteves *et al.*, 1998).

This study suggests that fertilisation influenced by high levels of nicotine exposure might be impaired since AR, which is an important step during *in vivo* and *in vitro* fertilisation, is adversely affected by high levels of nicotine exposure. This observation might prove as an additive factor to the poor fertilising ability of spermatozoa of smokers observed in previous studies (Shi *et al.*, 2001). In addition, the suppression of the sperm motility with increasing nicotine exposure and decrease in percentage of motile sperm fractions results in reduced number of spermatozoa reaching the oviductal ampulla and hence reduces the chances of fertilisation.

In conclusion, this study has supported the hypothesis that high levels of nicotine exposure might prove as an additive factor to reduced fertilisation capacity of human spermatozoa by decreasing motility, viability and inducing a premature AR. A decrease in sperm kinetic parameters, viability and ability of spermatozoa to achieve zona pellucida fusion during fertilisation was observed in the high-exposure treatment groups. Sperm motility parameters showed that spermatozoa had a low progression and lowered vigorous movement, which might have negatively affected the AR. The unaffected motility parameter in the low treatment group is in agreement with previous studies (Reddy *et al.*, 1995). The result of this study is in accordance with other studies that conclude that while nicotine levels found in the seminal plasma of smokers might not adversely affect semen parameters, high levels of nicotine exposure may certainly be an additive factor to the impediment of male fertility and certainly cigarette smoke with all its components combined is detrimental to an individual's health and should be strongly discouraged by all healthcare practitioners.

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