The effect of Methamphetamine on the Blood-Testis Barrier

By

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DECLARATION

I, Omer Saleh Zabida, hereby declare that "**The effects of Methamphetamine on the Blood-Testis Barrier**" is my own work and has not been submitted for any degree or examination in this or any other university. All the resources that I have used have been indicated and fully acknowledged by complete references.

Full name: Omer Saleh Omer Zabida Date: 28th of November 2018

Signed 

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ABSTRACT

Introduction

The blood-testis barrier (BTB) is formed by tight junctions between adjacent Sertoli cells. The barrier formed by these tight junction helps to create a specialized environment for spermatogenesis and provide an immunological barrier to protect developing germ cells. Methamphetamine (Meth) is known as neurotoxin however, its effects on the male reproductive system, especially on Sertoli cells and, the BTB are not well established. Therefore, this study aimed to determine the effects of Meth on the TM4 mouse testis Sertoli cell line and on the integrity of the BTB permeability.

Materials and Methods

This study investigated the effect of selected concentrations of Meth (0.1 μM, 1 μM, 10 μM, 20 μM and 100 μM) on TM4 mouse testis Sertoli cell line for 24 until 96 hours, using two treatments: an "acute" study (24 hrs exposure) and a "chronic" study, where treatment occurred on a daily basis over 96 hrs. The following parameters were investigated: viability, cell proliferation, mitochondrial activity, monolayer permeability.

Results

Although Meth slightly suppressed viability statistically at 100 μM (24 hrs, acute study) and again at 1 μM and 10 μM (72 hrs), these changes to viability fall within the normal control range. During chronic treatment, viability was only slightly suppressed at 1 μM and 10 μM (48 hrs). Both these incidents were also well within the normal range of viability to TM4 Sertoli cells. The data suggest that both acute and chronic Meth treatment of Sertoli cells had little to no effects on the viability of TM4 Sertoli cells.

Proliferation (rate of cell division) was suppressed in the acute study only at 48 hrs after withdrawal of Meth. The TM4 Sertoli cells recovered by 96 hrs. In the chronic study this effect was more pronounced and suppression only occurred at 10 μM, 20 μM and 100 μM concentration and persisted to 96 hrs.

Acute Meth exposure (24 hrs) suppressed mitochondrial activity (MA) at 20 μM and 100 μM (24 hrs). Thereafter, Meth had no statistical suppressive effects. Chronic exposure on MA also, showed the same pattern as in the acute study. However, slight statistical significant suppression of MA only occurred at 100 μM (96 hrs).

Acute treatment of monolayers TM4 Sertoli cells resulted in a dose-related decrease of TEER (increase permeability) at 24 hrs. After the withdrawal of Meth, this trend was reversed to show a dose-related increase in TEER (decrease permeability) which persisted to 96 hrs. Chronic treatment of monolayers TM4 Sertoli cells resulted a dose-related decrease in TEER throughout the study. The study showed clearly that the presence of Meth causes an increase permeability across the *in vitro* BTB model.

Conclusion

In summary, although Meth at physiological (blood plasma) concentration is not toxic to TM4 Sertoli cells, it was able to suppress cell division in both acute and chronic studies. Furthermore, Meth also tended to suppress MA at higher concentrations.

Also, Meth increase the permeability across the *in vitro* BTB, indicating that the specialized ionic environment, crucial for spermatogenesis, may be compromised in both occasional Meth users and in addicts.

The data, thus, suggest a mechanism for Meth-induced male infertility. Further, a Meth-induced decreased in Sertoli cells of seminiferous tubules may also decrease the optimum capacity for spermatogenesis, leading to a decrease sperm count.

Secondly, the increased permeability across the BTB, suggest a compromised luminal environment for spermatogenesis further contributing to a decrease in count sperm numbers. Thus, for the first time we show that these Meth effects both contribute towards a decrease sperm count, which is well known to the one of the main contributor to both male infertility and male sub-infertility.

KEYWORDS

Methamphetamine

Male reproductive system

Blood-testis-barrier

Sertoli cells

Cell viability

Proliferation

Trans-endothelial electrical resistance (TEER)

LIST OF ABBREVIATIONS AND SYMBOLS

5-HT	Serotonin
ABP	Androgen-binding protein
AJ	Adherens junction
ANOVA	Analysis of variance
ATCC	American type culture collection
ATP	Adenosine triphosphate
BCRP	Breast cancer resistance protein
cm	Centimeter
CNS	Central nervous system
Cntrl	Control
CO_2	Carbon dioxide
CT	Connective tissue
Cx	Connexins
DA	Dopamine
DAT	Dopamine transporters
DHT	Dihydrotestosterone
DMEM	Delbecco's modified eagle medium
DMSO	Dimethylsulphoxide
DOPAC	Dihydroxyphenylacetic acid
E	Epinephrine
EBD	Evans Blue Dye
ELISA	Enzyme-Linked Immunosorbent Assay
ES	Ectoplasmic specializations
ESAM	Endothelial cell-selective adhesion molecule
EtOH	Ethanol
FAC	Focal adhesion complex
FSH	Follicle stimulating hormone
GDP	Guanosine diphosphate
GLUT-1	Glucose transporter-1
GuK	Guanylate kinase
HBMVEC	Human Brain Microvascular Endothelial Cell
hBMVECs	Human Brain Microvascular Endothelial Cells

hCG	Human chorionic gonadotropin
HS	Horse serum
ICMART	International Committee for Monitoring Assisted Reproductive Technology
KO	Knockout
LHD	lactate dehydrogenase
MA	Mitochondrial activity
MAGUK	Membrane-associated guanylate kinase
MAP	Mitogen-activated proteins
MDH	Mitochondrial dehydrogenase
Meth	Methamphetamine
mg	Milligram
MIF	Müllerian-inhibiting factor
MIS	Mullerian-inhibiting substance
ml	Millilitre
MRP	Multidrug resistance-related proteins
N	sample number
NE	Norepinephrine
O_2	Oxygen
PBS	Phosphate buffered saline
PCNA	Proliferating Cell Nuclear Antigen
P-gp	P-glycoprotein
pH	logarithmic scale used for measuring acidity and basicity
P-value	Probability-value
ROS	Reactive oxygen species
rpm	Revolutions per minute
SA	South Africa
SACENDU	South African Community Epidemiology Network on Drug Use
SA-MRC	South African-Medical Research Council
SCCx43	connexin-43
SEM	Standard error of the mean
SSC	Spermatogonial stem cell

TB	Trypan Blue
TEER	Transendothellial electrical resistance
TGF	Transforming growth factor
TJ	Tight junction
TM4	Mouse testes Sertoli cell
TNF-α	Tumor necrosis factor- α
VMAT-2	Vesicular monoamine transporter-2
WC	Western Cape
ZO-1	Zonula occludens-1
ZO-2	Zonula occludens-2
ZO-3	Zonula occludens-3
α	Alpha
β	Beta
γ	Gamma
Δ	Delta
μM	Micro Molar
Ω	Ohms
%	Percent
˚C	Degrees Celsius
<	Less than symbol

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CHAPTER 1

1. Introduction

1.1 Prevalence of Methamphetamine abuse

The global production and trafficking of Methamphetamine (Meth) has risen to unprecedented levels and the use of this illicit central nervous system (CNS) stimulant is said to have 15-16 million users worldwide (Hobkirk et al., 2015). Meth is the second most extensively abused illegal drug globally after cannabis (Lin et al., 2014; Watt et al., 2014). According to the World Drug Report, global Meth confiscations quadrupled from 2008 to 2012 (Hobkirk et al., 2015). The number of countries that reported Meth as a cause of concern includes South Africa, Australia, Japan, the Czech Republic, New Zealand, Thailand, parts of North America (Plüddemann et al., 2010), other regions include Mexico, South America, the Middle East, the Arabian Peninsula and Asia (Schepers et al., 2003).

Meth use in South Africa (SA) was spurred on due to socio-economic and political changes that occurred at the end of apartheid (Watt et al., 2014). It is thought to have become popular between Cape Town in 1985 to 1999 (Charles, 2014) and has since become the drug of choice. Since 1999, the decay of communities within the Western Cape as a result of Meth addiction and abuse has been evident along with the rate of crime, theft, and child and partner abuse (Hobkirk et al., 2015).

Historically, Meth use in Africa has been low traditionally, however, by 2004 the numbers of patients reporting Meth abuse became more significant. In 2006, 73% of adolescents admitted for drug counselling in Cape Town (South Africa) reported Meth as their primary or secondary drug of choice (Plüddemann et al., 2010). In comparison to the rest of SA, Cape Town has the highest level of drug-related crimes and school violence (Charles, 2014). A survey conducted in Cape Town, found that Meth commonly known as ‘tik’ comprises 58% of substance abuse among adolescents who left school (Watt et al., 2014).

A study from 2002-2015 in subjects from rehabilitation facilities 42.3% reported ‘tik’ as their primary drug (Watt et al., 2014). In drug rehabilitation centres, Meth

abuse admissions sharply increased from 0.3% in 2002 to 42% in 2006 and began a slow decline to 33% by 2013 (Hobkirk et al., 2015). Recently, however, the South African Community Epidemiology Network on Drug Use (SACENDU), a research unit of the South African-Medical Research Council (SA-MRC) reports that in the Western Cape Province (WC) 37% of drug abusers report Meth as their primary substance in the period of July-December 2015, an increase from the January-June 2015 report of 35% (Dada, et al., 2016).

1.1.2 Historical overview of Methamphetamine

Meth is an amphetamine derivative that was first synthesized by Dr Nagayoshi Nagiai of Tokyo Imperial University in 1888 by reducing ephedrine with hydriodic acid and red phosphorus (Logan, 2002). There are a few derivatives from which Meth can be produced: phenyl-2-propanone, ephedrine and pseudoephedrine as well as from the plant 'ma-huang' (Mafunda, 2012). These recipes and methods for preparing precursors are readily available on the internet which has influenced its popularity (Logan, 2002).

Meth was patented in 1920 and marketed as 'Methedrine', an anorectic (Logan, 2002) and in 1932 as 'Benzedrine' an over the counter inhaler for nasal congestion (Mafunda, 2012). Later the drug was also used for a number of other ailments: narcolepsy, attention deficit disorder, eating disorders, obesity, depression and attention deficit hyperactivity disorder (Logan, 2002). In 1938 the first report of Meth psychosis and addiction was reported (Person et al., 2005). During World War II the drug was distributed to Japanese Kamikaze pilots, German and American soldiers as a performance enhancer, to keep troops awake and alert during combat. After the war, Meth supplements were made available to the Japanese public, this resulted in the first Meth epidemic which would later spread worldwide (Kingsberg, 2013). The supply of amphetamines to soldiers still continues today, during the Gulf war pilots were issued with 'GO pills' (5mg of d-amphetamines every 4 hrs) to combat fatigue (Logan, 2002).

1.1.3 What is Methamphetamine

Meth is a white crystalline powder (see fig 1.1) that easily dissolves in water or alcohol. This odourless and bitter tasting drug is a powerful and extremely addictive CNS stimulant (Thanoi & Thanoi, 2011; Charles, 2014; Watt et al., 2014). Meth is the common systemic name given for N, α-dimethyl phenethylamine which may also be referred to as methyl amphetamine, phenylisopropylmethylamine and desoxyephedrine. Meth belongs to the class of drugs known as amphetamines. Amphetamine is a shortened name for α-methylphenethylamine of which Meth is the N-methyl derivative (Mafunda, 2012). The addition of the *N*-methyl group to the basic structure of amphetamine is believed to give Meth its lipophilic properties (Kirkpatrick et al., 2012). Meth has many colloquial names, for instance, crystal meth, crystal, ice, speed, whiz and crank and here in Cape Town known as "tik" (Logan, 2002). The names usually refer to the illicit preparations of the drug as opposed to its pharmaceutical chemical counterparts. The ticking sound that is heard while Meth is smoked, has given rise to its common name 'tik' (Watt et al., 2014).

Figure 1. 1: Meth crystals (http://www.atlantaduilawyer.com/possession-of methamphetamine-georgia/).

1.1.4 Route of administration

The majority of Meth abusers ingest Meth intra-nasally (snorted), while other methods include: oral ingestion of Meth capsules or wrapping the drug in a toilet paper in order to smoke it, inhaling the fumes or taking it intravenously (Logan, 2002). In most cases Meth abusers use a 'lolly' (a glass tube with a round closed end which resembles a lollipop) to heat the Meth and inhale its fumes. Smoking is the most common route of Meth administration especially in hot spots like Cape Town (Hobkirk et al., 2015). Intranasal inhalation and smoking of Meth are seen as safer routes of administration as they decrease the risk of transmission of blood-borne diseases however, they still expose the body to Meth leading to drug-related complications (Harris et al., 2003).

1.1.5 Metabolism of Methamphetamine

Metabolism of Meth takes place in two phases and the breakdown products are usually conjugated to glucuronic acid before it is excreted in the urine (Logan, 2002). The first phase of Meth metabolism usually involves N-demethylation of most of the Meth to amphetamine which will be broken down to other products namely norephedrine and *p*-hydroxyamphetamine (Logan, 2002). The detectable breakdown products of Meth found in urine are: amphetamine 4-7%, *p*-hydroxymethamphetamine, and *p*-hydroxyamphetamine of which 15% and 1% respectively (Schepers et al., 2003). Alternatively, a study by Cook et al. (1993) reported that 30-54% of the drug was excreted unchanged and 10-23% as amphetamine in the urine. Ninety percent of the Meth dose ingested is excreted in the urine in its metabolized or un-metabolized forms (Schepers et al., 2003). The by-products of Meth breakdown are pharmacologically active and may induce their own effects all around the body. Some common drugs may be broken down into *d*- or *l*-methamphetamine and amphetamines such as Selegeline (Deprenyl®), a drug prescribed for Parkinson's disease and Famprofazone (Gewodin®) used as an analgesic (Logan, 2002).

Depending on the route of administration the metabolism and elimination of the Meth differs. Various elimination half-lifes for different methods of Meth consumption have been reported. However, generally, intravenous Meth injections

have an elimination half-life of 11-12 hours, intranasal half-life of 10.7 hours, Meth smoked half-life of 10.7 hours and orally half-life of 10 hours (Cruickshank & Dyer, 2009; Harris et al., 2003; Schepers et al., 2003). An inter-species difference in terms of half-life also exists, for example between humans and rats, 10-12h and 1h respectively (Segal & Kuczenski, 2006). Therefore, caution should be taken when comparing rats to humans as their Meth elimination half-life are significantly different.

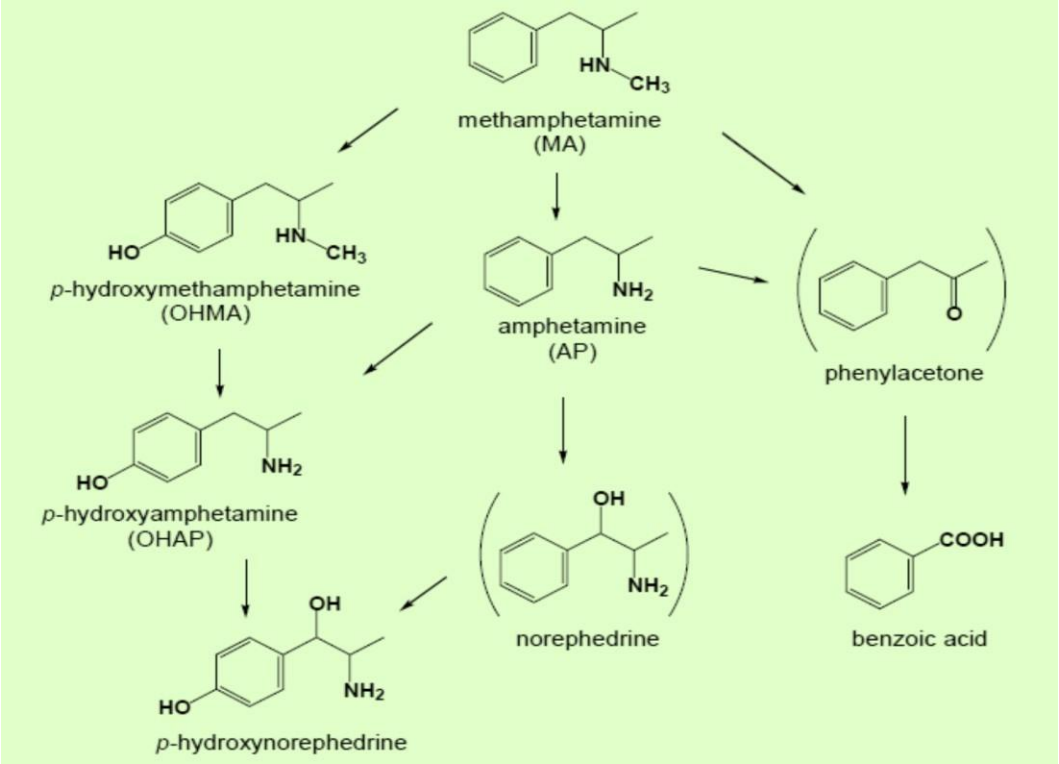


Figure 1. 2: Metabolism of Meth within the rat liver (Caldwell et al., 1972).

1.1.6 Biological concentration of Methamphetamine

Due to the haphazard behavior of Meth abusers, it is difficult to simulate the time and exact dose that is taken each time as the reports from the users are not reliable when trying to draw up accurate generalizations on Meth exposure. Overall, the Meth concentrations of addiction ranged between 0.13 and 11.1 μM. This study also found much higher Meth values (up to 84 μM) in post-mortem subjects and it is most likely that they died due to Meth toxicity (Melega et al., 2007).

1.1.7 Effects of Methamphetamine

The effects of Meth are reported to occur in a dose-dependent manner (Yamamoto et al., 2002; Thanoi & Thanoi, 2011). They can be grouped into psychological effects which are associated with the CNS and its components, and physical effects which involve the entire body. They may also involve neurotransmitter fluctuations as a result of changes that take place in the CNS (Darke et al., 2008).

The CNS effects of Meth abuse are so pronounced because of Meth's ability to competently cross the blood-brain barrier (BBB) due to its property of lipid solubility (Mafunda, 2012) and this applies to the blood-testis barrier (BTB) as well. The most prominent ills associated with Meth abuse are mental health problems including depression, anxiety, psychosis, violent behavior (Plüddemann et al., 2010) agitation, aggression, euphoria and mania (Watt et al., 2014). The mechanism underlying Meth-induced neurotoxicity is believed to be a combination of cellular toxicity, oxidative stress, and apoptosis, but this still needs to be fully clarified (Ramirez et al., 2009; Lin et al., 2014).

The physical effects are the signs and symptoms seen communally, reported and categorized as 'fight-or-flight' responses. The peripheral harms of Meth are so marked due to its agonistic properties with α, β1 and β2 adrenergic receptors (Logan, 2002). Meth produces its characteristic fight-or-flight effect through α-receptors these include extreme vigilance and physical activity, bronchial muscle dilation, vasoconstriction, pupillary dilation (mydriasis), a decrease in appetite, increased libido, skin tremors, weight loss, bladder contraction, hyperthermia and hypernatremia (Logan, 2002; Watt et al., 2014).

Meth may also induce cardiopulmonary complications, increasing the heart rate and blood pressure of users regardless of the route of administration (Darke et al., 2008; Watt et al., 2014). Therefore, users who have heart problems or cardiovascular disease may be at a higher risk for myocardial ischemia and infarction as well as arrhythmia's (Darke et al., 2008). Meth is also known as the 'sex drug' as in male's ejaculation is delayed, orgasm intensity is enhanced, and libido is increased, however, at high doses, most males lose interest in sexual activity as they fail to achieve orgasm (Cruickshank & Dyer, 2009; Logan, 2002).

1.1.8 Methamphetamine's mechanism of action

Meth is a sympathomimetic drug; these are drugs which mimic endogenous neurotransmitters of the sympathetic nervous system by interacting with their receptors (Schepers et al., 2003). Meth is able to interact with these receptors due to its structure which is extremely similar; some examples include catecholamines, norepinephrine (NE), dopamine (DA), epinephrine (E) as well as serotonin (5-HT) (Logan, 2002; Mafunda, 2012).

The potent effects of Meth at dopaminergic axons are due to its ability to substitute itself in place of DA at its transporters and receptors. Meth promotes the release of DA from their stores in nerve terminals and inhibits its entry into the axon, preventing its storage within vesicles (synaptosomes) and furthermore, blocks its attachment to its common transporters, vesicular monoamine transporter-2 (VMAT-2) and dopamine transporters (DAT) (Vearrier et al., 2012). Meth also easily traverses the nerve plasma membrane due to its lipophilic properties and stimulates the release of DA out of the nerve terminals into the synapse, while also impairing the storage of DA in synaptosomes (Vearrier et al., 2012). Therefore, Meth is able to enhance the release of DA and other neurotransmitters, specifically NE, at their synapses resulting in characteristic effects, the anorectic and 'alertness' (DA) seen in users, as well as the hyper-locomotive effects (NE) (Logan, 2002). The imbalance of these neurotransmitters produces the characteristic CNS symptoms seen in abusers.

1.2 Overview of the male reproductive system

The male reproductive system consists of internal organs which include the accessory glands, ejaculatory ducts, vas deferens, seminal vesicles, urethra, epididymis and external genitalia; the penis and scrotum (see fig 1.3) (Barrett, et al., 2010; Seeley et al., 2014). The male reproductive system allows for the manufacturing and distribution of a number of products, for example, production of essential hormones like testosterone, production, maturation, and release of spermatozoa (Pearce, 2014). The main aim of this system is to produce spermatozoa and deposit it into the female vagina with the aim of procreation. Spermatozoa are

produced in the testes, matures as it moves through the male and female reproductive system with the aim of fertilizing an egg (Pearce, 2014).

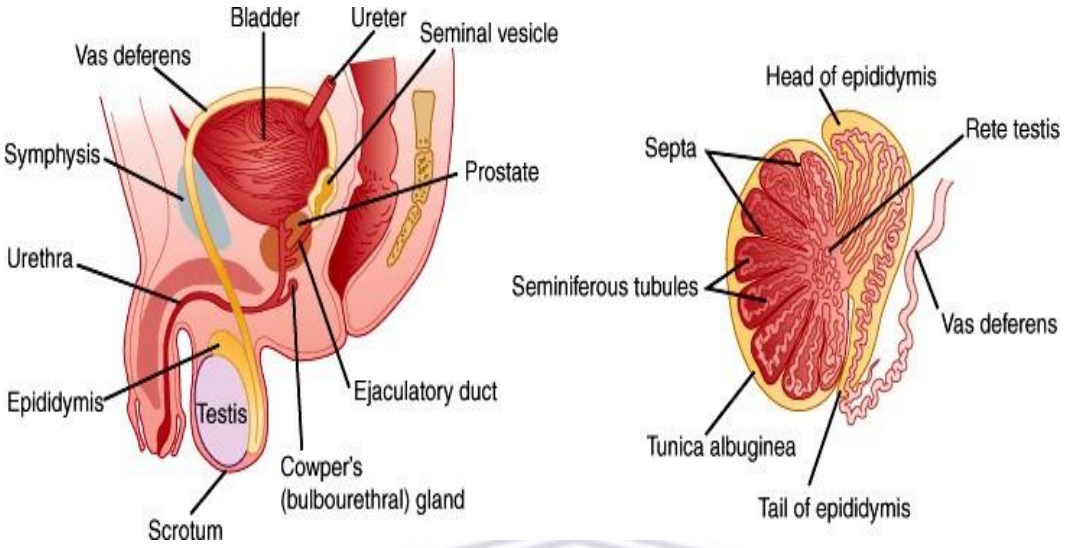


Figure 1. 3: Anatomical features of the male reproductive system and a cross-section of the testes and epididymis showing the duct system by which sperm travels (Barrett, et al., 2010).

1.2.1 The testis

The testes are a pair of specialized oval-shaped organs suspended by the spermatic cord found in a specialized fibromuscular sac known as the scrotum. The testes are responsible for the production, maturation, and protection of developing sperm cells (Pearce, 2014; Opuwari, 2009).

The testes are surrounded by several outer layers consisting of skin (scrotal sac), a serous membrane (Tunica vaginalis), a dense connective tissue layer, a vascular layer as well as two muscles which assist with temperature control (the dartos and cremaster muscles). The *tunica vaginalis* forms the serous covering of the testes and is believed to be derived from the peritoneum which precedes the descent of the testes during male development. The *tunica vasculosa* consists of blood vessels connected by areolar tissue which form a plexus on the inner side of the *tunica albuginea* as well as around the structures it covers (Pearce, 2014). The *tunica albuginea* is a dense layer of connective tissue (CT) with abundant collagen fibers, as well as contractile and smooth muscle cells (Svingen & Koopman, 2013). The

tunica albuginea allows for blood and lymphatic vessels to pass through to the testes and is responsible for regulating blood flow, sperm movement through rhythmic contractions and inter-testicular pressure (Svingen & Koopman, 2013). These fibers are continuous with the testis and some extend into the testis and form fibrous partitions termed *septa*. These *septa* divide the testes into several lobules containing the convoluted seminiferous tubules (see fig 1.3) (Martini et al., 2012).

1.2.2 The seminiferous tubule

The testes contain a number of duct systems including the seminiferous tubules, and then to the rete testis, these tubes turn into the ductus efferents, which emerge from the dorsal part of each testis and fuse to form the ductus epididymis, which helps to mature and store spermatozoa. The seminiferous tubules are about 80 cm long, slender tubules that line and occupy each lobule (Martini et al., 2012). They are surrounded by a delicate CT capsule and the space between tubules is filled by interstitial tissue, containing the Leydig cells and blood vessels (Pearce, 2014). Within these tubules, production and development of male sex gametes take place and therefore these tubules and their cells play a crucial role in maintaining male fertility.

Seminiferous tubule epithelium is containing a complexly stratified epithelium with two distinguishable cell populations: the non-proliferating, supporting cells (Sertoli cells) and germ cells with different stages of maturation (see fig 1.4).

1.2.3 Sertoli cells

Sertoli cells (SCs) as they are commonly known, also known as nurse cells, ramifying cells, supporting cells or sustentacular cells (Pearce, 2014). SCs are named after their discoverer Enrico Sertoli (1842-1910) (Opuwari, 2009). SCs play an important role in nourishing, protecting and providing structural support to developing spermatogonia and ensure spermatogenesis takes place (Hess, 1999; Pearce, 2014). These cells are both columnar and stellate in shape and they line and extend the entire height of the seminiferous epithelium into the lumen of the seminiferous tubule (Hess, 1999; Opuwari, 2009). SCs are specialized epithelial cells that lie side-by-side forming a ring within the seminiferous tubules. The

spermatocytes in the various stages of development are tucked between adjacent Sertoli cells and remain this way until they develop into spermatids (Hess, 1999; Seeley et al., 2014). SCs also have a unique distribution of organelles which relates to its regional function and relationship with germ cells in that area (Opuwari, 2009).

The SCs safely envelopes the migrating spermatocytes until spermiogenesis and spermiation take place (Hess, 1999). As the spermatogonial germ cells migrate they undergo further division while still in contact with Sertoli cells. Also, SCs have apical and ventral processes which extend between and around every germ cell (Opuwari, 2009). Seminiferous tubules fluid is secreted by the SCs and assists with flushing germ cells into the epididymis for storage and further maturation (Seeley et al., 2014). During spermiation the communication between SCs and germ cells is broken and gap and adherens junctions (AJs) are severed. Tight junctions (TJs) and gap junctions (GJs) are formed between two cells of SC and developing germ cells. This allows for communication between them; transfer of wastes, and delivery of essential nutrients. SCs also provide chemical stimuli important for spermatogenesis (Martini et al., 2012).

Further, the barrier formed between SC TJs to restrict molecule movement into the intra-tubular fluid and this creates a specialized environment that varies significantly from the composition of blood plasma and testicular lymph (Cheng & Mruk, 2012).

The SCs (see fig 1.4) also play the role of 'nurse cells' as they provide nourishment to the maturing germ cells. They utilize specialized transporters and receptors to help with the uptake of nutrients to supply the germ cells, for example, the glucose transporter-1 (GLUT-1); a transporter responsible for glucose movement assists the Sertoli cell by providing the glucose to be metabolized to lactate which is then transported to the germ cells (Seeley et al., 2014). Lactate is produced via glycolysis under the regulation of SCs; glucose is metabolized to lactate and then pyruvate under the influence of lactate dehydrogenase (LDH). The reason for SCs action in glucose metabolism to lactate is that germ cells are unable to efficiently metabolize glucose and become low glucose metabolism *in vivo*, the SCs help to metabolize glucose to lactate for the germ cell consumption (Pearce, 2014; Reis et al., 2015).

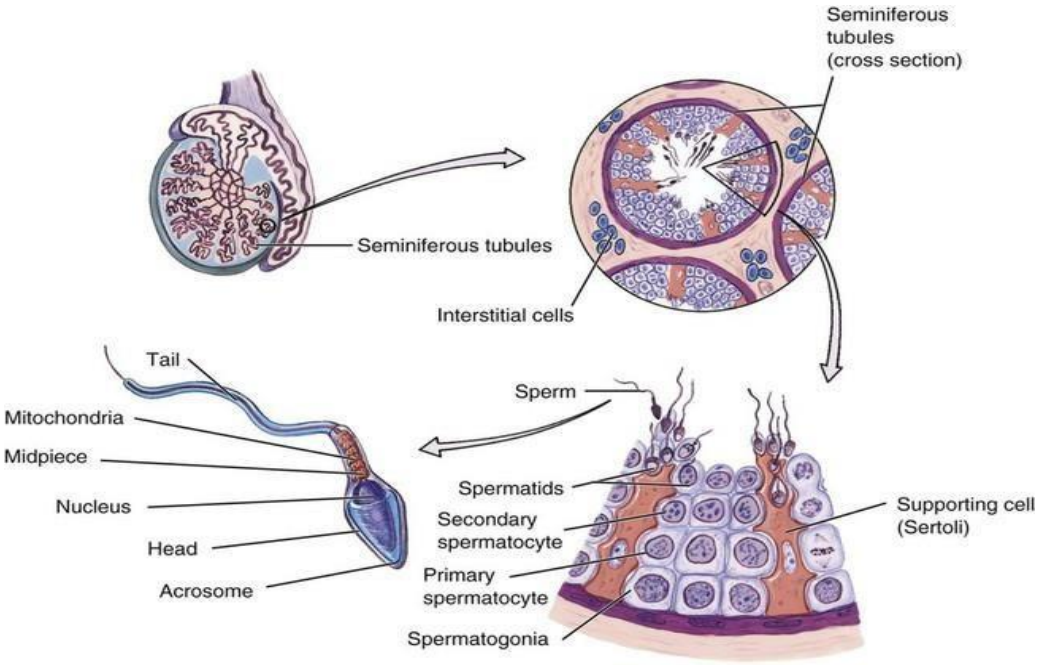


Figure 1. 4: Section of Seminiferous epithelium where developing germ cells are seen invested in the Sertoli cell cytoplasm as they move from the basal lamina to the lumen of the tubule and the different stages of Spermatocyte growth are visible (https://nursekey.com/reproductive-system-3/).

Important phagocytic functions are also performed by the SCs. As sperm cells transition from early round spermatids to late spermatids or spermatozoa, they shed their excess cytoplasm by a process called spermiation. The SCs take up the excess cytoplasm and phagocytose similarly to an immune cell (Martini et al., 2012). The Sertoli cells are also responsible for disposing of defective germ cells and they help to create an immunological barrier that protects developing germ cells (Hess, 1999) from the immune system (Reis et al., 2015). This barrier inhibits immune cells found in the extracellular fluid from reaching the 'sperm factory' found in the germinal epithelium of the seminiferous tubule. The absence of this barrier would otherwise allow the recognition of the spermatocyte antigens as foreign and stimulate the production of anti-sperm antibodies; a response of this nature may lead to male infertility (Pearce, 2014; Seeley et al., 2014).

The SCs are also the site of hypothalamus-pituitary hormone action for the control of spermatogenesis by both testosterone and follicle-stimulating hormone (FSH). Distinct receptors for each of these hormones are found on SCs. FSH directly activates intracellular signaling pathways that lead to the secretion of paracrine factors which promote spermatogenesis (Chen & Liu, 2015).

SCs secrete many substances under the influence of FSH including transferrin and lactate. They are responsible for the crucial transport of copper (Cu2+), iron (Fe3+), dihydrotestosterone (DHT), testosterone and many other substances to the developing spermatogonia in order to fuel spermatogenesis (Pearce, 2014). Another substance secreted by Sertoli cells is androgen-binding protein (ABP). This protein binds to androgens like testosterone to help maintain very high levels of testosterone within the seminiferous tubule. The high local concentration of testosterone in the seminiferous tubules are 100 times that of blood and is critical for maintaining sperm manufacturing within the tubules. Testosterone is lipid soluble and could easily diffuse out of the tubule and be lost into the blood had it not been for ABP, testosterone itself promotes ABP production (Sherwood, 2010).

In addition, SCs secrete other substances including inhibin, Mullerian-inhibiting substance (MIS), somatomedin C, transforming growth factor (TGF) alpha and beta. These cells also play an important role in contributing to the basement membrane by secreting certain proteins; type IV collagen and laminin (Pearce, 2014). Hormones like 'inhibin' are secreted from Sertoli cells and act as regulators of FSH secretion in a negative feedback fashion (Seeley et al., 2014).

1.2.4 Spermatogenesis

The process of spermatogenesis is a continuous, productive biological process of spermatozoa formation from spermatogonial stem cells (SSC) to mature spermatozoon's that takes place within seminiferous tubules of the testes over a period of time (Hess, 1999). Spermatogenesis is a 14 step procedure in the mouse testis seminiferous epithelium (Lee, 2013). Spermatogenesis involves a few biological processes: proliferation spermatogonia by continual mitotic divisions, replication of genetic material (chromosomes), and genomic recombination via cross-over, meiotic division to yield haploid primary 2nd spermatocytes and terminal differentiation of spermatids into spermatozoa (Hess, 1999). Therefore, three phases can be extrapolated each associated with a particular cell type: 1. proliferation of spermatogonia by mitosis, 2. reduction-division involving spermatocytes and 3 differentiations; spermatids are differentiated into spermatozoa (see fig 1.5) (Hess, 1999; Reis et al., 2015).

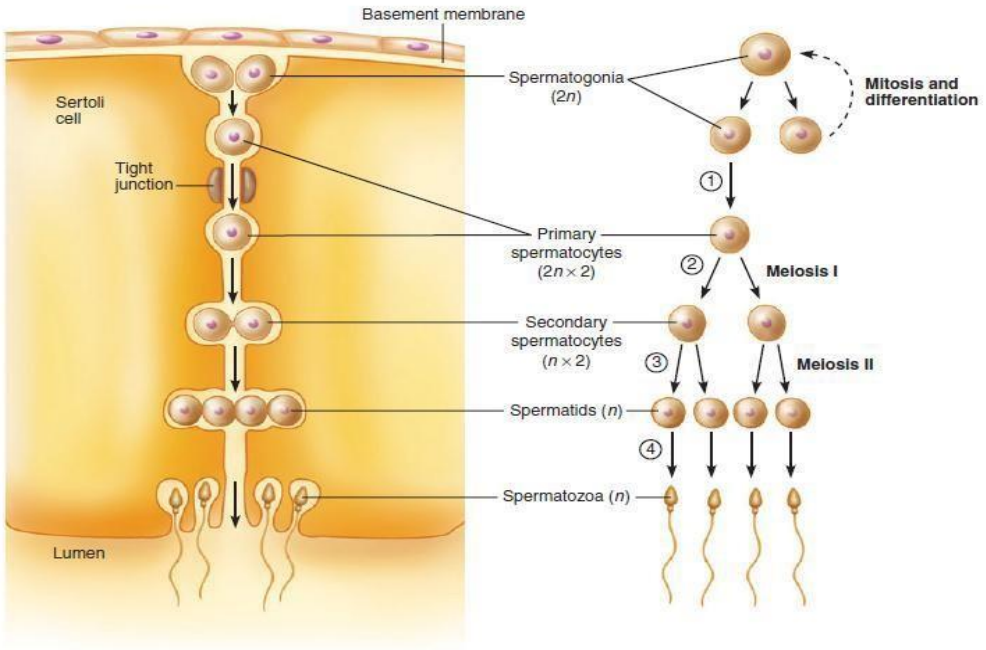


Figure 1. 5: Process of spermatogenesis. A single diploid (2n) primary spermatogonium cell develops and differentiates to yield 4 haploids (n) spermatozoa (Stanfield, 2012).

Spermatogenesis takes place within the widespread seminiferous tubules network of the testis. Seminiferous tubules are lined by specialized epithelium and comprise a fluid-filled lumen into which spermatozoa are released. The seminiferous epithelium comprises of two specialized cell types: somatic cells and germ cells (Hess, 1999). The somatic cells are called Sertoli cells; they nest the germ cells in its cytoplasm as it develops and ascends from the basement membrane of the tubules to the adluminal compartment. The germ cells have to ascend through tight junctional complexes which are formed between Sertoli cells before they reach the lumen (Hess, 1999).

Spermatogonia are the first cells in the spermatogenesis process; they are simple, self-renewing cells found along the base of the seminiferous epithelium. They are referred to as self-renewing cells as they produce more stem cells as well as cells that are committed to spermatogenesis (Hess, 1999; Chen & Liu, 2015). These type A- spermatogonia may undergo proliferation to replenish the germinal epithelium, or they may divide by meiosis and ascend up to the lumen by producing type B-spermatogonia (Hess, 1999; Opuwari & Monsees, 2014; Chen & Liu, 2015).

Type B-spermatogonia divides last and produce the cells which pass through the Sertoli-Sertoli junctions and begin the migration to the lumen where they are called preleptotene spermatocytes (Hess, 1999). These are cells which are smaller than an erythrocyte and undergo reduction-division or meiosis: a specialized form of the cellular division for gametes which halves the number of chromosomes (Hess, 1999). In preleptotene cells, DNA synthesis takes place and is followed by prophase 1 which may last up to 3 weeks (Hess, 1999). Once chromosomes have unraveled into thin filaments homologous pairs form and are bound together by synaptonemal complexes (Hess, 1999).

The cells continue to divide into pachytene spermatocytes which are much larger than their preleptotene counterparts. Pachytene spermatocytes begin as a small cells however, their nuclei enlarge immensely as their chromosomes shorten and thicken, paired chromosomes cross-over and genetic recombination takes place (Hess, 1999). In diplotene spermatocytes the chromosomes pair into individual chromosomes by loosening the synaptonemal complexes followed by diakinesis; the nuclear membrane disintegrates and chromosomes condense (Hess, 1999). Meiosis takes place rapidly, during the first phase small secondary spermatocytes (2N) are produced which then rapidly divide again into even smaller haploid (N) cells called round spermatids (Hess, 1999). Once spermatocytes have differentiated into spermatids they will undergo further differentiation into immature spermatozoa during the final phase of spermatogenesis known as spermatogenesis. Spermatozoa lose contact with the seminiferous epithelium and enter into the fluid of the lumen where they will travel via the tubules of the testes to the epididymis where they are periodically stored till they are ejaculated. Further, maturation also takes place after they enter the female reproductive system (Martini et al., 2012).

1.3 The Blood-testis barrier

The BTB is established in rats by 21 days postpartum (Mok et al., 2011). The environment created by the BTB is a unique *milieu* that has characteristic amounts of peptides, ions, steroid hormones, growth factors, amino acids, glucose and glycolytic intermediates (Haverfield et al., 2014). This specialized environment differs from blood plasma, testicular lymph and intertubular fluids (Reis et al., 2015).

The BTB is formed between adjacent Sertoli cells near the basement membrane and is comprised of TJs, basal and apical ectoplasmic specializations (ES), GJs and desmosome-like junctions (Cheng & Mruk, 2012; Su et al., 2011). The highly specialized ultrastructure of the BTB is due to the combination of all of the junctions as well the basal ES which make the BTB one of the tightest blood-tissue barriers in the mammalian body (Cheng & Mruk, 2012). In addition, the TJs also divide the seminiferous tubule into a basal compartment and adluminal compartment. Within the basal compartment, spermatogonia are found and mitosis takes place while meiosis and spermatogenesis take place within the adluminal compartment (Martini et al., 2012; McCabe et al., 2016).

1.3.1 Tight junctions (TJs)

Sertoli TJs play an essential role in spermatogenesis as they form an impermeable seal between adjacent SCs, thereby protecting and separating germ cells in basal and adluminal compartments and form an integral part of the BTB (McCabe et al., 2016; Cheng & Mruk, 2012). TJs join adjacent Sertoli cells just above the spermatogonia that lie on the basal membrane (Opuwari, 2009), a specialized extracellular matrix (Cheng & Mruk, 2012). During spermatogenesis, the TJs between Sertoli cells assemble below the mitotically active spermatogonium, while actin filament bundles of two Sertoli cells tightly formed as a sandwich between cisternae of the endoplasmic reticulum and disassemble above it allowing it into the special environment they create (Cheng & Mruk, 2012).

Sertoli cell TJs include occluding, claudins, junctional adhesion molecules (JAMs) (McCabe et al., 2016) as well as cytoplasmic proteins which connect the transmembrane proteins to the actin cytoskeleton. Occludins and claudins are transmembrane proteins which are tethered to the cell cytoskeleton by protein complexes called zonula occludens (ZO)-1, -2 and -3 (see fig 1.6) (Moroi et al., 1998; Shi et al., 2014).

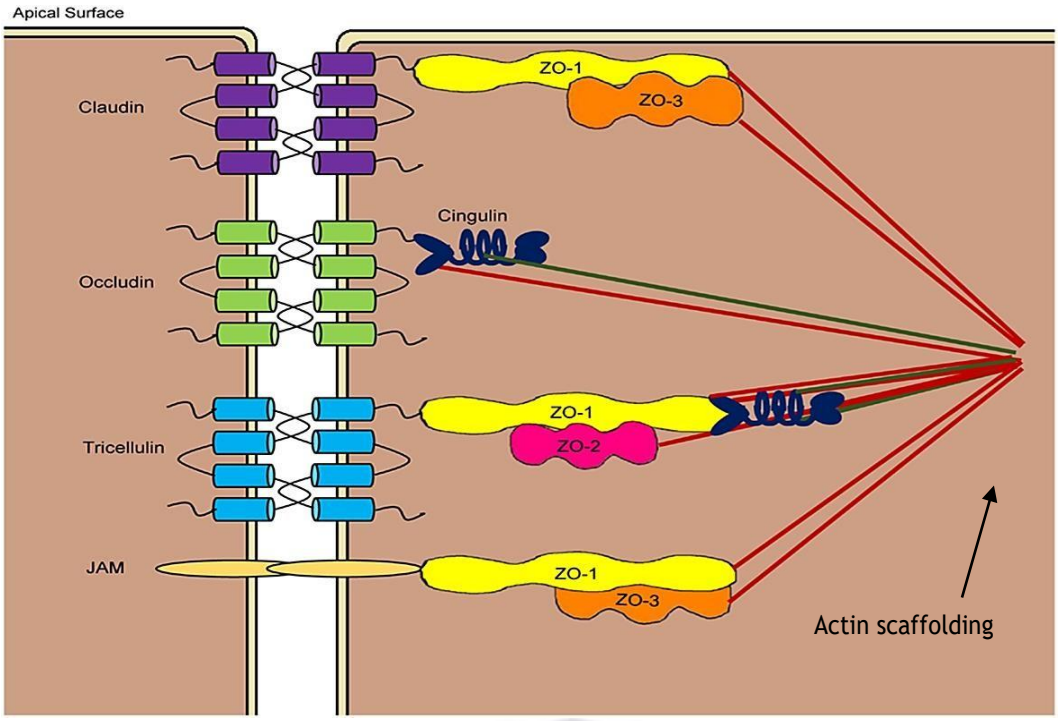


Figure 1. 6: Tight junction proteins, occluding, claudins and junctional adhesion molecules (Robinson et al., 2015).

1.3.1.1 Claudins

Claudins are 20-27kDa phosphoproteins which have four transmembrane domains, two extracellular loops and a short carboxyl tail (Morrow et al., 2010). Claudins form the principal seal of TJs and are essential for TJ establishment (Shi et al., 2014). There are 24 different known claudins (Shi et al., 2014) of which claudin -1, -3, -5, -8, -11, -12, and 19 (McCabe et al., 2016) are all said to play roles in the testes. Claudin-3 and -5 are expressed at stage VIII of spermatogenesis and claudin-11 is expressed throughout the process (Morrow et al., 2010). It has been hypothesized that claudin-3 forms part of 'new' TJs formed underneath migrating spermatocytes whereas claudin-11 is the widespread form 'old TJs' found in almost all stages of spermatogenesis which are found above migrating spermatocytes and are targeted for disassembly (Stanton, 2016).

Although claudin-11 can be detected in the brain, it is principally expressed in Sertoli cells (Shi et al., 2014). Analysis done indicated that claudin-11 formed part of tight junction strands between Sertoli cells indicating its role as part of the BTB (Tsukita et al., 1999) and claudin-11 expression peaks at the time of BTB formation

(Shi et al., 2014). Claudin-11's imperative role is further endorsed by knockout (KO) studies in mice where a mature BTB is not established and germ cells are full of apoptotic bodies (Shi et al., 2014). Claudin-11 KO mice are infertile (Stanton, 2016).

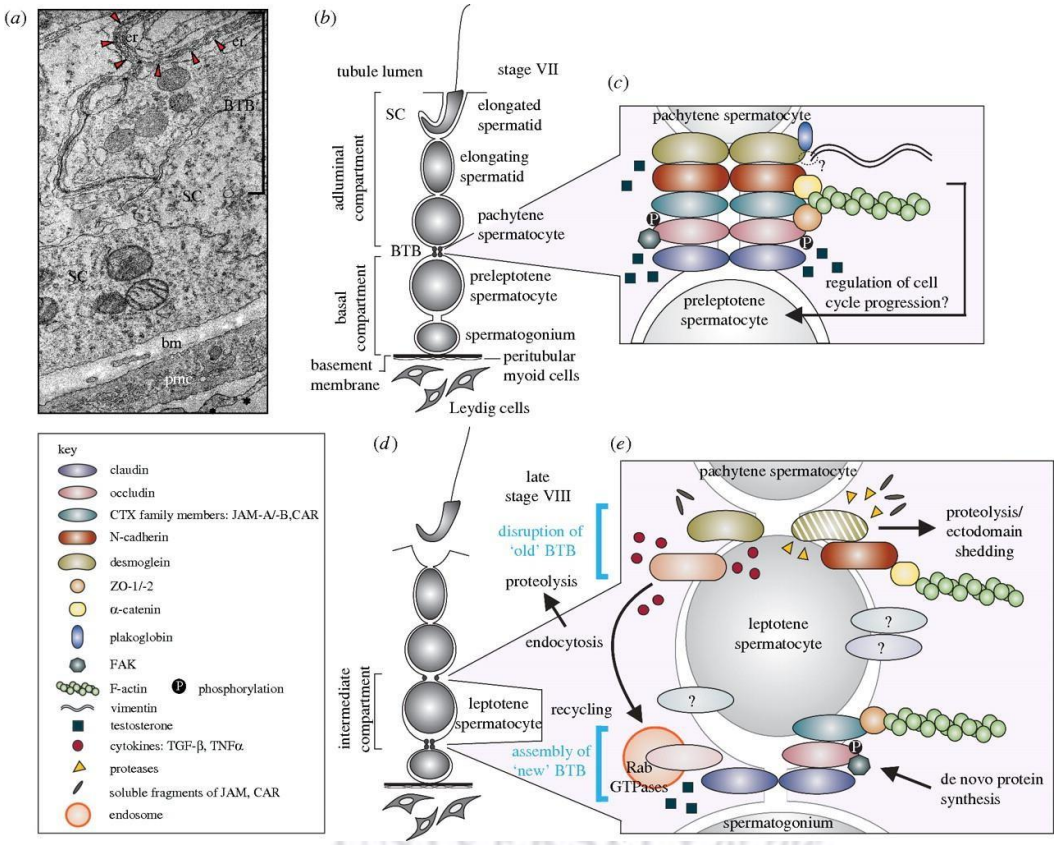


Figure 1. 7: Schematic diagram of the BTB and the migration of the developing spermatogonia through the BTB and the junctional proteins involved, a) and b) is a diagrammatic representation of the way a spermatogonium divided by mitotic division and sent through the germinal epithelium from the basement to the lumen, c) is an expansion of the TJ structure, d) and e) describe the regulation and development of intermediate spermatids from the basement membrane cross the BTB (Mruk & Cheng, 2010).

1.3.1.2 Occludins

Occludins are 60-65kDa integral membrane proteins and were the first proteins localized to TJs (Tsukita et al., 1999; Chiba et al., 2008; Morrow et al., 2010). Occludins have two extracellular loops (ECL), a short cytoplasmic (intracellular) turn, with a short cytoplasmic N-terminus (amino) and a long cytoplasmic C-terminus (Tsukita et al., 1999; Chiba et al., 2008) and four transmembrane domains (Morrow et al., 2010). The first ECL-1 has a substantial amount of tyrosine and glycine residues (60%) (Tsukita et al., 1999) and mediates occludins' adhesive

abilities. The C-terminal associates with zonula occludens (ZO-1) which joins it to the cytoskeleton (Chiba et al., 2008) via its guanylate kinase domains. Occludin may be involved in TJ formation along with fibroblasts but occludin is not necessary for the maintenance or the establishment of TJ as demonstrated by occludin-knockout (KO) studies (Morrow et al., 2010).

Occludin can be detected in the testes by immunofluorescence studies and can be detected in all stages of the seminiferous epithelial cycle in most animals including mice and dogs. However, in rats, occludin expression is stage-specific as it is not detected in stage VIII (Shi et al., 2014). Occludin is not expressed in the seminiferous tubules of humans and guinea pigs (Moroi et al., 1998; Shi et al., 2014). The absence of occludin in spermatogenesis develops slowly and gradual changes are seen with ageing. These changes include seminiferous tubules lacking germ cells and at about 40-60 week apoptotic tubules are seen but they retain their Sertoli cells (Shi et al., 2014).

1.3.1.3 Zonula Occludens proteins

Zonula Occludens (ZO) proteins or membrane-associated guanylate kinase (MAGUK) proteins are characterized by multiple postsynaptic density protein-95 large disc domains (PDZ) which are (ZO-1 binding domains), a SH3 domain (Src homolog-3 domain) and a Guanylate kinase-like (GuK) domain (Tsukita et al., 1999). These domains enable multiple protein-protein interactions (Hawkins & Davis, 2005), mediating specific binding to the carboxyl-terminals of cytoplasmic proteins (PDZ), binding signalling proteins and cytoplasmic elements (SH3) and catalysing ATP-dependent transformation of guanosine monophosphate (GMP) to guanosine diphosphate (GDP) (Cardoso et al., 2010). The SH3-GuK domains are further involved in TJ and AJ protein binding. The MAGUK proteins are responsible for TJ placement recognition proteins as well as supporting proteins for signal transduction (Cardoso et al., 2010). TJ-associated sub-membrane proteins such as ZO are found in three forms: ZO-1, zonula occludens-2 (ZO-2), and zonula occludens-3 (ZO-3). ZO's regulate the effectiveness of the TJ (Abbott et al., 2010).

ZO-1 is 220kDa cytoplasmic or plaque phosphoproteins which are joined to the C-terminus of claudins by its PDZ, associated to JAM via its PDZ 2 and 3 domains

and to occludin via the GuK domain. ZO-1 acts like a bridge joining these proteins to the actin cytoskeleton via its C-terminal. ZO-1 has a peculiar distribution due to its affinity (N-terminal) for the carboxyl terminal of occludin and α-catenin and the association of its carboxyl-terminal with Actin filaments. Therefore, ZO-1 forms a critical link in TJs and without it, TJs cannot exist. Evidence has also linked ZO-1 to connexin-43, a component of gap junctions, and at Afadin, another cytoplasmic accessory protein (Tsukita et al., 1999).

ZO-2 is smaller than ZO-1 with a molecular mass of 160kDa. It has similar homology to ZO-1 but at its GuK domain, its amino-terminal is followed by a short proline-rich carboxyl domain (Tsukita et al., 1999). In contrast to ZO-1, ZO-2 is not only found in TJs but in AJs as well. ZO-2 is similar to ZO-1 as it can also cross-link occludin and α-catenin and it also binds to transcription factors. ZO-2 can also form heterodimers with ZO-1 using its second PDZ domain (Tsukita et al., 1999).

ZO-3 is a 130kDa protein and is the smallest out of these three. ZO-3 was initially thought to only be a protein that co-precipitates with ZO-1 and or ZO-2 complex as it has similar homology, the difference being that ZO-3 has a proline-rich domain between PDZ-2 and PDZ-3 (Tsukita et al., 1999). Experimental evidence has shown that ZO-3 can bind to ZO-1 (Tsukita et al., 1999) and occludin as well, but not to ZO-2.

1.3.1.4 JAMs (Junction associated membrane proteins)

JAMs are 40kDa (mw) proteins forming part of the Immunoglobulin (Ig) superfamily (Tsukita et al., 1999; Chiba et al., 2008). This molecule has a single transmembrane domain (Tsukita et al., 1999), two extracellular domains, and a short C-terminal cytoplasmic loop. The JAM family has two subgroups: JAM-1 (JAM-A, JAM, 106-antigen, and F11R), JAM-2 (JAM-B, VE-JAM, h-JAM-2, and mJAM-2), JAM-3 (JAM-C, h-JAM-3, and mJAM – 2) (Liu et al., 2012). JAM -1, -2 AND -3 form group one AND have class II PDZ domains which bind at their C-terminal ends and directly interact with ZO-1 and PAR-3 (Chiba et al., 2008). Endothelial cell-selective adhesion molecule (ESAM), and JAM-4 are part of the second subgroup and have class I PDZ domain-binding motifs at their C-terminus

(Chiba et al., 2008). JAM-A takes part in cell-cell adhesion, organizing and formation of TJs along with occludin and claudin and is predominantly involved in BTB barrier function in endothelial and epithelial cells. JAM-B and JAM-C canbe found on round spermatids, where JAM-C is pivotal in their polarization (Chiba et al., 2008).

AF-6 (also called Afadin) is a 205kDa protein with two Ras- stimulating domains, one PDZ domain and regions of homology with Myosin V and Kinesin (Liu et al., 2012). The Ras domains interact with ZO-1 and in the event that these domains are activated the interaction between AF-6 and ZO-1 is inhibited. This may indicate a role in the modulation of TJ pathways that incorporate Ras (Hawkins & Davis, 2005; Liu et al., 2012).

1.3.2 Ectoplasmic specializations

ES co-exist with TJs within the BTB near the basement membrane (Cheng & Mruk, 2012). The ultrastructural characteristics of the ES consist of tightly packed, hexagonally arranged, actin bundles inserted between the cisternae of the endoplasmic reticulum and the Sertoli cell plasma membranes (O'Donnell et al., 2000; Mruk & Cheng, 2004; Cheng & Mruk, 2012). The proteins that make up the ES (α-actinin, fimbrin, vinculin, epsin, gelsolin, and ZO-1) are said to also make up the adherence junctions in other tissues as well (Mruk & Cheng, 2004) however, the molecular identity of these proteins is not well understood as most of the characterization has been done using electron microscopy (O'Donnell et al., 2000). Dysfunction of ES is a possible target for male contraception as their disruption leads to depletion of germ cells (Mruk & Cheng, 2004).

Two types of ES are found in the testes namely the basal and apical ES (Cheng & Mruk, 2012; Mruk & Cheng, 2004). Basal ES are restricted to the area between adjacent Sertoli cells close to and incorporated into the BTB close to the basement membrane whereas the apical ES is found in the adluminal compartment between Sertoli cells and elongating spermatids (Lee & Cheng, 2004; Mruk & Cheng, 2004; Wong, et al., 2008; Cheng & Mruk, 2012). TJs, GJs, focal adhesion complex (FAC) proteins and AJs can be localized at apical ES as it is believed they are essential for the restructuring process which takes place during spermatogenesis (Wong et al.,

2008). Three multi-protein complexes have been identified in ES namely: the integrin/laminin, the nectin/afadin/ponsin/ and the cadherin/catenin complexes (Lee & Cheng, 2004).

The cadherin/catenin complex that is found in the testis is one of the best studied AJ structural complexes out of the three complexes which have been defined (Lee & Cheng, 2004). Cadherins have cytoplasmic domains which associate with catenins and extracellular domains of AJs for assembly via homophilic interactions. There are many different cadherins: N (neural) and E (epithelial)-cadherins are the more classic forms (Lee & Cheng, 2004) whereas other less common forms include Cadherin-5 or VE-cadherin which is expressed in vascular tissues and is essential for their integrity. N- and E-cadherin are both found in the seminiferous epithelium of rats and mice. Cadherin-E is said to be expressed more by germ cells than Sertoli cells and the preconceived notion that only Sertoli cells provide adhesion was dropped (Lee & Cheng, 2004). N-cadherin can be found at apical and basal ES and N-cadherin are considered a structural component of the BTB (Lee & Cheng, 2004). Vimentin, an intermediate filament of the cytoskeleton has been shown to be associated with as much as 50% of N- and E-cadherin (Lee & Cheng, 2004).

Catenins, are intermediary proteins located in the paracellular junctions. Catenins are involved in signaling pathways those involved in testis development and function to secure the cadherin complex to the actin cytoskeleton (Shi et al., 2014). There are four types of catenins: alpha (α), beta (β), delta (δ) and gamma (γ). Beta- and gamma-catenins connect to the actin cytoskeleton, via alpha-catenin (Lee & Cheng, 2004). β - Catenin is connected to the cell membrane along with N-cadherin and therefore is an integral component of the BTB (Lee & Cheng, 2004). In the absence of these proteins, cadherins will not be able to bind to the cytoskeleton (Shi et al., 2014).

The integrin/laminin complex was one of the first AJ structural proteins localized to the apical ES (Lee & Cheng, 2004). Integrin is composed of α and β subunits of which eight β and 18 α have been identified. Integrin functions as a transmembrane protein receptor (Lie et al., 2013) and is limited to the focal adhesion site between cells and the ECM (Lee & Cheng, 2004). Integrins mediate cell-cell and cell-matrix adhesion by binding laminins (Lie et al., 2013). Laminins are heterodimers which

are made up of α, β and γ chains (Lee & Cheng, 2004). Laminin is an integral binding partner integrin and is said to be confined to the basement membrane in most epithelia. Recently, laminin γ3 has been localized to the testis along with β-1 integrin (Lee & Cheng, 2004).

1.3.3 Gap junctions (GJs)

GJs also referred to as connexin-43 (Cx 43) or GJA1, which is the principal testicular GJ protein. The role cx-43 plays are said to regulate Sertoli cell maturation and spermatogenesis (Sridharan et al., 2007; Cheng & Mruk, 2012). The role of GJs as a signaling platform between cells has been investigated. GJs form intercellular pathways that join the cytoplasm of adjoining cells and allows the passage of substances smaller than 1.5kDa (Sridharan et al., 2007). Substances that can pass through GJs to coordinate processes include inorganic ions, cyclic nucleotides, siRNA duplexes, glucose, polypeptides, and ATP in response to changes in their surroundings (Cheng & Mruk, 2012). Each GJ is composed of connexons or hemichannels which connect adjacent cells. These connexions are made up of smaller units called connexins (Cx), for example, Cx43, Cx33, and Cx26 which are all localized to the testes (Sridharan et al., 2007; Cheng & Mruk, 2012). A hexamer of Cx makes up a hemichannel, these hemichannels may be homo- or heterotypic (Cheng & Mruk, 2012).

1.3.4 Desmosomes

Desmosomes are flexible and adhesive cell-cell intermediate filament-based anchoring junctions found predominantly at the Sertoli-spermatid interface as well as at the BTB between Sertoli cells (Cheng & Mruk, 2012). The proteins that make up the desmosome can be divided into three groups: the plakin family; desmoplakin, the desmosomal cadherins; desmogleins and desmocollins and the armadillo (arm) proteins; plakoglobin and plakophilin (Cheng & Mruk, 2012). The deletion of anyone one of these components can have substantial effects. For example, the knockdown of desmoglein-2 and desmocollin-2 reversibly disrupted and destabilized Sertoli cell TJ permeability and caused the mislocalization of essential

junction proteins (occludin, ZO-1, CAR, proto-oncogene tyrosine-protein kinase Src(c-SRC)) (Cheng & Mruk, 2012).

1.3.5 Influx and efflux BTB pumps

The BTB has 'gatekeeper' functions to prevent harmful substances from entering the adluminal compartment (Reis et al., 2015) Numerous drug transporters are known to associate with TJs. These transporters regulate the substances which are allowed to enter the specialized immune-privileged environment of the adluminal compartment of the testes. Influx pumps are expressed by Sertoli cells and help regulate the traffic of various substances required for spermatozoa maturation including testosterone. Examples of influx pumps include: anion and cation transporters (Reis et al., 2015) A few efflux pumps and their roles in the BTB will be briefly discussed.

P-glycoprotein (P-gp) is an ATP-powered pump vastly expressed in Sertoli cells and is responsible for moving harmful substances against their concentration gradients P-gp is an efflux pump and has recently been shown to associate with a number of TJ proteins including basal ES and other BTB constituent proteins: occludin, claudin-11, ZO-1 and JAM (Su et al., 2011) usually when the cells are under attack (Reis et al., 2015). P-gp is said to enhance the protective functions of spermatogenesis at the BTB.

Multidrug resistance-related proteins (MRP) are efflux proteins of which three family members are known for their drug transport capabilities. One form of MRPs is known to associate with the Sertoli cells and is also extensively found in many tissues is MRP-1. MRP-1 is said to play an essential role in keeping drugs out of the seminiferous tubules and protecting the BTB (Reis et al., 2015). MRP-1 mutant mice exposed to an anticancer drug was found to have impaired spermatogenesis and an increased number of prematurely released spermatocytes (Reis et al., 2015).

Another efflux pump is breast cancer resistance protein (BCRP). BCRP is extensively expressed in the testes and is said to play a vital role in inhibiting phytoestrogenic compounds from acquiring access to Leydig and Sertoli cells.

BCRP is also essential for hormone transport, germ cell development and maturation (Reis et al., 2015).

1.3.6 Restructuring of the BTB during spermatogenesis

BTB restructuring involves a number of biochemical structures and processes: GJs, desmosomes, steroids like testosterone and estradiol, non-receptor protein kinases and cytokines (Cheng & Mruk, 2012). Cytokines and testosterone are the driving forces behind BTB restructuring they are actively involved in all aspects of BTB restructuring. The process of restructuring involves endocytosis, recycling, degradation, transcriptional regulation, cytoskeletal remodeling and intracellular cascades (Lie et al., 2013).

The BTB is restructured at stage VIII-IX of the seminiferous epithelium cycle to facilitate the migration of preleptotene spermatocytes at the basal compartment as it develops into leptotene, zygotene and pachytene spermatocytes and spermatids (round and elongated) found in the apical and intermediate compartments (Haverfield et al., 2014; Yazama, 2008). Without germ cell migration males are deemed infertile (Li et al., 2009; Su, et al., 2011). During BTB restructuring (VII-VIII in the rat) the highest expression of androgen receptors (ARs) takes place and therefore these stages are considered androgen-dependent.

The restructuring of the BTB takes place at the basal region of the Sertoli cell junctions and at the apical ES between Sertoli cells and elongating spermatids in the adluminal compartment (Li et al., 2009). Emerging evidence has been found indicating the involvement of GJs and desmosomes with the assistance of non-receptor protein kinases: c-Src, c-yes and focal adhesion kinase (FAK) which are said to help coordinate protein redistribution of TJs and basal ES (Cheng & Mruk, 2012). The integrin/laminin complex of the apical ES (Li et al., 2009), the hemidesmosomes and BTB form an axis which facilitates barrier restructuring. Briefly, just prior to spermiation laminin at the apical ES is cleaved, the biologically active forms are able to down-regulate β-1 integrin affecting hemidesmosomes and disrupting BTB integrity by decreasing occludin (Lie et al., 2013).

Cytokines have been shown to disrupt the BTB *in vivo* and *in vitro*, affecting germ cell numbers and also affects the BTB's integrity (Li et al., 2009). The cytokines involved include but are not limited to tumor necrosis factor- α (TNF-α), interferon-γ (IFN- γ), TGF-β2 and -β3, interleukin (IL) -12 and interleukin-1α (Cheng & Mruk, 2012). The above-listed group is said to regulate BTB TJ permeability under pathological and non-pathological conditions. Cytokines are also said to be regulated stage-specifically to help supervise spermatogenesis. These cytokines may be similar by their effects are mediated by different pathways. Two pathways have been proposed as TJ dynamic forces: TGF-β3/MEKKs/p38 mitogen-activated proteins (MAP) kinase and the TNF-α/integrin-linked kinase/p130 Crk associated substrate (CAS)/ MAP kinase signaling. In the cadmium male contraceptive model the TGF-β3/p38 pathway was implicated (Wong et al., 2008).

The transcriptional regulation of BTB function is also regulated by cytokines and testosterone which elicit effects on the levels of BTB TJ proteins (Lie et al., 2013). Cytokines and testosterone also regulate TJ integrity by the effects of cytokines on AR expression and on testosterone production (Li et al., 2009). Cytokines modulate steroidogenesis in Leydig cells and the manifestation of ARs in Sertoli cells. The method by which steroidogenesis is controlled involves TNF-α and IL-1α. GJs may also play an essential role in mediating the effects of cytokines (Li et al., 2009).

The cytoskeleton is made up of F-actin, tubulin and vimentin molecules (Lee & Cheng, 2004). The cytoskeleton plays a pivotal role during the BTB restructuring as it functions in conferring not only cell shape and cell rigidity, but also facilitating movement by interaction with adaptor and signaling molecules as well as polymerization and de-polymerization reactions (Lee & Cheng, 2004). Once more cytokines (IL-1α) play vital roles, IL-1α induces the destabilization of the actin bundles making up basal ES, cell junctions and the BTB as well as the formation of budding branches on actin filaments. Other cytokines also involved in cytoskeletal remodeling are TGF-β3 and TNF-α (Lie et al., 2013).

Endocytosis, recycling and degradation of TJ proteins also take place under the influence of cytokines and testosterone. The internalization of these TJ proteins results in the restructuring of the BTB by early endosomes via the clathrin-mediated mechanism (Lie et al., 2013). The caveolin-mechanism has also been suggested, as

an increase in occludin's association with clathrin and rab-11 takes place at the same time. Testosterone is involved with the recycling of internalized proteins to the plasma membrane below migrating spermatocytes. Cytokines have opposing effects on BTB restructuring are TGF-β2 and -β3 which perturb TJ integrity due to protein degradation (occludin), while IL-1α decelerates the dynamics of occludin break-down (Lie et al., 2013).

A number of theories have been proposed on how exactly BTB restructuring takes place via the zipper theory, the intermediate compartment theory, the stress/repetitive removal of membrane segment theory and the junction restructuring theory (Yazama, 2008; Opuwari, 2009). A theory proposed by Dym and Fawcett in 1970 suggested that the BTB is a dynamic interface and it must 'open' to accommodate the elevation of spermatocytes for germ cell migration and 'close' to maintain BTB integrity (Yazama, 2008).

Russel in 1977 proposed the' intermediate compartment theory'; he showed that a third compartment, apart from the basal and adluminal compartments, was formed and thus explained germ cell migration without the BTB being compromised (Opuwari, 2009). The stress repetitive removal of membrane segment theory says that 'stress' induces changes in the BTB TJs resulting in changes in terms of shape, orientation, proliferation and breakdown but was unable to explain exactly what triggered the stress (Opuwari, 2009). Lastly, the junction restructuring theory proposed that the timely passage of germ cells was made possible by cell junctions going through periods of assembly and disassembly. The theory furthermore, stated that for example TJ proteins occludin and claudin (Tsukita et al., 1999) must progressively break to allow the timely passage of preleptotene spermatocytes across the BTB (Opuwari, 2009).

Another theory is that the BTB uses a unique assembly, disassembly and reassembly to permit disruption of AJs to facilitate germ cell migration while maintaining BTB integrity. The BTB is made up of a number of TJs and AJs which are co-localized but do not interact with one another. These proteins are structurally linked by their adaptors, namely catenins and ZOs which attach them to the actin cytoskeleton (see fig 1.7). Therefore, under physiological conditions, these proteins engage and reinforce the BTB, but when they are exposed to toxicants, like adjudin,

they induce restructuring via the AJs that leads to germ cell loss in the seminiferous epithelium (Yan & Cheng, 2005).

The BTB is not compromised during the migration of spermatocytes as it is able to maintain its immunological barrier throughout spermatogenesis. The most accepted BTB theory involves intermediate compartment theory. The BTB is important to establish the division between two compartments: the basal which contain spermatogonia and an adluminal compartment containing the later differentiation stages of germ cells, the BTB also has to maintain the microenvironment of the seminiferous tubules.

1.3.7 Models to study the blood-testis barrier

Most models proposed were found to be expensive or too difficult to routinely implement in the lab for BTB studies or culture methods which did not mimic the *in vivo* BTB structures and processes sufficiently. Therefore, the need for a model which is economical and repeatable is highly desirable required (Li et al., 2009). Generally, two methods of studying the BTB exist: *in vitro* and *in vivo.* The *in vitro* model is based on the primary culture of Sertoli cells independently as this method is inexpensive and quick to complete and analyze. Many researchers have used this method to study the structure and function of the tight junctions which make up the BTB (Shi et al., 2014). However, it has proved challenging to test the BTB function as a co-culture of Sertoli cells and germ cells. The co-culturing with germ cells has not worked as getting the germ cells to complete meiosis has not been successful in all studies reported (Shi et al., 2014). Therefore, this model is insufficient for an in-depth study of the structure, function and regulation of the BTB.

The majority regarding the understanding of aspects of the BTB and its junction components, for instance, ES regulation, is due to *in vitro* studies using co-culture of germ cells and Sertoli cells or Sertoli cells only (Mruk & Cheng, 2004). An *in vitro* study showed that the addition of testosterone and FSH in co-cultures can increase the number of spermatids that bind to Sertoli cells (Mruk & Cheng, 2004). Another study on N-cadherin and vinculin showed that their levels increased when Sertoli cells and germ cells were cultured together (Mruk & Cheng, 2004). Therefore, *in vitro* models are able to provide information on Sertoli cells and the

BTB but the *in vitro* culturing methods as they are not always able to simulate completely the *in vivo* conditions (Cheng & Mruk, 2012).

A study by Legendre et al. (2010) developed an *in vitro* model which replicates the composition, organization, barrier properties and spermatogenesis functions of the *in vivo* rat BTB using a 3D two-tier chamber of testicular cells isolated from 18-day old rats. This model is able to show the effects of a substance on spermatogenesis as well as detect the effect of chemical compounds on the barrier (Legendre et al., 2010). Several studies which made use of bicameral chambers were able to demonstrate differentiation of germ cells into haploid cells *in vitro.* However, these 3D systems are not useful for studying the physical and chemical functioning of the BTB (Legendre et al., 2010).

The *in vivo* models involves the use of mice with two basic approaches; transgenic and knockout mice (mutant rodent models) (Mruk & Cheng, 2004). Other BTB models involve androgen suppression, adjudin, the cadmium model, the glycerol model and oestrogen models, (Mruk & Cheng, 2004). As these models take place within an organism they more closely represent what will happen in a human subject. As for permeability, *in vivo* Evans Blue Dye (EBD) was one of the models that were used to investigate permeability of the BTB (Hamer et al., 2002).

1.4 Adjudin model

Adjudin is a potential male contraceptive that is able to disrupt germ cell attachment in the seminiferous epithelium. A study by Mok et al. (2011) showed that low-dose adjudin cleared all spermatocytes except the spermatogonial stem cells found in the basal compartment. The seminiferous tubules could be repopulated by germ cells once the adjudin was metabolized by the host and fertility was reinitiated. But in high-doses adjudin, fertility failed to recover and the population of spermatogonia within the seminiferous tubules was similar to the low dose group with the presence of spermatogonial stem cells, but no germ cells undergoing spermatogenesis (Mok et al., 2011).

1.5 Conditions associated with Sertoli cell dysfunction

During the development of the testes in the fetus, Sertoli cells secrete Müllerian-inhibiting factor (MIF) (Sherwood, 2010). This hormone is responsible for the regression of the Müllerian ducts which form the uterus and uterine tubes in females. Inadequate secretion of MIF may result in underdevelopment of these ducts and failure of the testes to descend into the scrotum.

Cryptorchidism is the failure of one or both of the testes to descend into the scrotum by the time of birth (Seeley et al., 2014). Typically, the testes are lodged in the abdominal cavity (cryptorchid) or the inguinal canal. The testis may be surgically moved to the scrotum. Testes that remain in the abdominal cavity may become cancerous and males who do not have this corrected before puberty will be infertile (Martini et al., 2012). In experimental models of cryptorchidism, the morphology and integrity of the BTB remain the same. Although the domino effect of cryptorchidism is the rapid loss of haploid germ cells and severe impairment of spermatogenesis (Yazama, 2008).

The BTB may also be studied by other mechanisms, like the different models proposed above, but also by gene mutation models. The TJs and other junctions forming the BTB are imperative to its functioning and maintenance of male fertility. Some KO models have found that offspring may be fertile regardless of the mutation but the tightness of the barrier, the proliferation and abilities of Sertoli cells and germ cells may all be affected in these models. Claudin-11 is essential for TJs throughout spermatogenesis, therefore, its depletion can be detrimental to the BTB. In a Claudin-11 KO study, mice were sterile due to the lack of TJs between Sertoli cells (Morrow et al., 2010).

Another study in the Sertoli cell connexin-43 (SCCx43) gap junction gene was knocked out. SCCx43KO mice have been shown to have an intact BTB but are infertile. The seminiferous tubule had a Sertoli only phenotype and only a few germ cells were present (Sridharan et al., 2007; Gerber, et al., 2016). The Sertoli cells continued to proliferate and heavily occupied the seminiferous tubule but they failed to mature. It was also reported that the type-A spermatogonia were arrested in their state and did not differentiate to initiate spermatogenesis (Cheng & Mruk, 2012).

1.6 Meth effects on reproductive function

1.6.1 Male infertility and Meth

The World Health Organisation (WHO) and the International Committee for Monitoring Assisted Reproductive Technology (ICMART) describe infertility as a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after a year or more of regular unprotected sexual intercourse (Opuwari & Monsees, 2014; Zegers-Hochschild et al., 2009). The reasons for infertility are credited to 35% in females, 30% in males, 20% in both males and female and while 15% is regarded as idiopathic. In one-third of couples said to be infertile the presence of a male factor is usually the cause (Fronczak et al., 2012) whereas in most African settings infertility is incorrectly blamed on the female partner (Shalaweh et al., 2015).

Male infertility may be associated with low sperm production, poor sperm motility, and abnormal sperm morphology. The primary cause of male infertility is seen as poor semen quality characterized by low sperm viability and motility (Opuwari & Monsees, 2014). Other factors which may be contributes to male infertility includes: marijuana use, anabolic-androgenic steroid use, cocaine, opioid narcotics and methamphetamine abuse, (Fronczak et al., 2012) while nutrition, socio-economic factors, lifestyle and exposure to environmental factors also can play a role in causing infertility (Gabrielsen & Tanrikut, 2016; Reis et al., 2015). Idiopathic causes have been added to excess reactive oxygen species (ROS) production by spermatozoa and inflammation of the genito-urinary tract (Opuwari & Monsees, 2014).

1.6.2 Effect of Meth on the male reproductive system

Meth is a known CNS toxin, however, also recognized as teratogenic and this is toxic to embryos (Lin et al., 2014; Yamamoto et al., 1999). Maternal Meth exposure in animal studies increases the risk of perinatal death, congenital abnormalities, and neurobehavioral impairments and leads to low birth weight (Cruickshank & Dyer, 2009). A few studies have outlined the effects of Meth on offspring and associated doses as little as 14mg/kg/day to cause skeletal malformations and 19mg/kg/day

with external malformations like cleft palate (Hammon & Griffin, 2007). Male mice treated with 15mg/kg/day of Meth failed to impregnate females although a significantly decreased number of vaginal plugs following 24 hours of cohabitation, the amount of intromissions, ejaculations and mounts over a 90-minute period was also decreased when compared to a control group (Hammon & Griffin, 2007).

Although, many factors can affect the male fertility, for example, increased testicular temperature, incomplete development of testis, environmental and nutritional factors and also endocrine disturbances, hormonal steroidogenesis and the regulation of testicular cells are some of the main causal factors (Edjenguele et al., 2014). Meth is also known to result in infertility by inhibiting the motility of sperm and by affecting the integrity of SCs tight junction (Sun et al., 2011) and also by decreasing sperm counts in rats (Gabrielsen & Tanrikut, 2016).

Meth also affects spermatogenesis by inducing apoptosis in cells involved in most stages of spermatogenesis (Thanoi & Thanoi, 2011; Alavi et al., 2008; Gabrielsen & Tanrikut, 2016) and compromises the antioxidant defense system of the testes (Lin et al., 2014). In a study to evaluate the effects of repeated of Meth dosing on apoptosis and proliferation of rats cell, Meth was found to significantly decrease proliferation and increase apoptosis, this was endorsed by the following observations: the presence of proliferating cell nuclear antigen (PCNA)-positive spermatogonia was at 95% and the presence of TUNEL-positive germ cells was also extremely high (Alavi et al., 2008). Studies by Yamamoto showed that Meth induces apoptosis in the seminiferous tubules of the testes (Yamamoto et al., 2002; Thanoi & Thanoi, 2011).

Spermatogenesis proteins, for example, the α-tubulin protein of the spermatozoa cytoskeleton, involved in differentiation is also altered when exposed to Meth (Sun et al., 2011). Studies by Yamamoto and colleagues in rats found decreased ejaculations, mounts and intromissions over a 90-minute period after Meth administration (Hammon & Griffin, 2007; Yamamoto et al., 1999).

A study by Lin et al. (2014) found that serum levels of testosterone were reduced across all time periods examined and testicular functions were also suppressed by the administration of Meth (Lin et al., 2014). These evidence are supported by

studies showing that Meth is known to affect the hypothalamic-pituitary-testicular axis by disrupting its functioning, damaging testicular structures, decreasing levels of the luteinizing hormone as well as damaging sperm (Fronczak et al., 2012; Yamamoto et al., 1999). Furthermore, Janphet et al. (2017) reported the effects of Meth has on DA and NE: Meth increased the levels of DA's metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) in the acute binge dose group whereas in chronic groups NE and its metabolite were significantly decreased. These alterations in testicular catecholamine's concentrations may lead to reproductive dysfunction (Janphet et al., 2017).

Although, the effects of Meth on the male reproductive system is a widely reported in studies on the effects of Meth on sperm motility, quality and morphology as well as decreases in serum testosterone and catecholamines, inducing apoptosis in the seminiferous tubules and adversely affect male fertility (Alavi et al., 2008; Fronczak et al., 2012; Hammon & Griffin, 2007; Janphet et al., 2017; Yamamoto et al., 1999), few studies have focused on its effect on the Sertoli cells. Although, the majority of studies have looked at Sertoli cell junctional components during assembly and disassembly of the BTB TJ, claudin-11 and occludin, no Meth studies on these crucial germinal structures have been reported.

Sertoli cells are extensively involved in germ cell development and therefore many studies are confirming changes in germ cell motility, spermatogenesis proteins, depletion of testosterone and androgen receptors then Sertoli cells, as well as the BTB, have to be affected. Therefore, this study proposes to determine the effect of acute and chronic exposure to Meth on the Sertoli cells and the BTB by determining the viability, cell toxicity, proliferation, and live cell average and cell permeability using a specific assay after acute and chronic Meth exposure.

Research aim and objectives

Aims

To analyse the *in vitro* acute (24 hrs) and chronic (96 hrs) effects of Meth using selected physiological parameters.

Objectives

In vitro analysis of Meth (acute and chronic exposure) on TM4 Sertoli cells in order to determine:

(i) Effects on Sertoli cell viability, toxicity and rate of cell division over 96 hrs, using the Trypan Blue exclusion assay.
(ii) Effects on Sertoli cell mitochondrial activity over 96 hrs, using XTT assay.
(iii) Effects on Sertoli cell monolayer permeability by using the trans-epithelial electrical resistance assay (TEER), over 96 hrs.

CHAPTER 2

Materials and methods

The *in vitro* study was carried out in order to investigate responses of an immortalized Sertoli cell line to acute exposure (24 hrs) and chronic exposure (daily: 24 – 96 hrs) to Meth (0.1 µM, 1 µM, 10 µM, 20 µM and 100 µM) and selected physiological parameters were investigated – namely, cell viability, cell toxicity, rate of cell division, mitochondrial activity and cell permeability.

2.1 TM4 Sertoli cell line

A TM4 Sertoli cell line was used for the purpose of the study (ATCC® CRL-1715) TM4 cells were cultivated at 37ºC and 5% CO_2, under standard aseptic work conditions. TM4 cells were cultured in Dulbecco's modified eagle medium (DMEM /F-12, Lonza S.A.), growth medium supplemented with 2.5% Fetal Bovine Serum (FBS, Gibco S.A.), 5% Horse Serum (HS, Lonza S.A) and 1% Penicillin (100 IU/ml, Sigma S.A) and Streptomycin (100 µg/ml, Sigma S.A).

2.1.1 Culture of TM4 cells

Cells were cultured in 75 cm² flasks, SPL, Korea, and allowed to reach 70% confluence and were passaged once this was reached. To remove the trypsin inhibitor in the growth medium, it was discarded and cells washed with 2 ml sterile phosphate buffered saline (PBS). Following this, 2 - 3 ml of 0.25% trypsin was added, and allowed to cover the surface of the flask and incubated at 37ºC until cells detached. The trypsinization of the cells took approximately 6 minutes and the detachment was monitored using an inverted microscope. To neutralize the trypsin, 2 - 3 ml of supplemented growth medium was added, and the detached cells were transferred to a 15 ml conical tube to be centrifuged at 2500 rpm for 5 minutes. Thereafter, the supernatant was removed, the cell pellet re-suspended in 5 ml complete growth medium. To further passage cells 1 ml (containing approximately – 10^6 cells) of the resulting suspension was transferred into a new 75 cm² flask, containing 10 ml complete growth medium. Passage numbers were recorded to

track the age and physiology of the cells. Additionally, cell morphology was observed and compared with cell viability.

2.1.2 Cell counting, viability and toxicity

Cell counting was performed using a Neubauer's hemocytometer. This was carried out by combining 40 µl of cell suspension with an equal volume of 2 % Trypan Blue (TB) and thereafter, transferring 10 µl of the mixture to both hemocytometer counting chambers.

The chamber was viewed under an inverted light microscope and the total number of dead and live cell counted for each experiment. Cell viability and cell toxicity was calculated according to equation 1 and 2.

1) The % cell viability was determined using the following equation:

$$\text{Cell Viability (\%)} = \frac{\text{No. of unstained cells (live)}}{\text{Total no. cells}} \times 100 \qquad \textbf{[Equation 1]}$$

2) The % cell toxicity was determined using the following equation:

$$\text{Cell Toxicity (\%)} = \frac{\text{No. of stained cells (dead)}}{\text{Total no. cells}} \times 100 \qquad \textbf{[Equation 2]}$$

2.2 Methamphetamine addition of the media

To prepare A 1M stock solution of Meth, 185.7 mg of Meth powder was weighted and dissolved in 1ml of PBS, the resulting stock solution was vortexed and then filtered through a syringe filter of pore size 0.45 µm (Cameo). Meth solution of concentrations (0.1 µM, 1 µM, 10 µM, 20 µM and 100 µM) were prepared by serial dilutions of the stock with complete media and these are used as treatment concentration for the cultured TM4 cells treatment groups. The control was made up with media only.

The TM4 cells were then seeded into 6 well plates. Media containing the different concentration of Meth was added to the cell cultures 24 hrs after seeding. Acute treatment was carried out for 24 hrs and thereafter, cells were only exposed to

culture media (supplemented growth media) until 96 hrs, while the chronic set of cells was treated with Meth until 96 hrs.

2.3 Principle of Trypan Blue assay

TB is a carcinogenic dye used to determine the amount of viable cells present in a sample. Non-viable cells absorb the TB due to their non-selective permeability, whereas viable cells do not absorb the dye, thus when viewed under a light microscope, non-viable cells stained blue while viable cells do not (Mascotti, 2000). Thus, the TB assay can determine both cell viability and cell toxicity by using the selective permeability of cells, non-viable cells retain and absorb the TB (purple-coloured), while the viable cells do appear as bright cells under high-power light microscopy (Mascotti, 2000; Strober, 2015).

2.3.1 The experimental treatment

2.3.1.1 Treating TM4 Sertoli cells with Meth

After determining the concentration of cells (cell number), TM4 cells were seeded at a density of 2×10^4 cells/well in a 6 well plate (SPL, Korea). Triplicate samples was used for each concentration. 24 hrs after seeding, cells were exposed to the selected concentration of Meth.

TM4 cells were acutely exposed (24 hrs) and chronically exposed by treating cells with Meth daily for 96 hrs. Control cells were exposed to standard supplemented DMEM. Throughout the experiment, the cells were incubated at 37°C and 5% CO_2. After treatment for the selected time periods (24, 48, 72 and 96 hrs) with the different concentrations of Meth, TM4 cells were trypsinated to perform the TB assay to determine cell viability assay.

2.3.1.2 Cell counting using a Neubauer's hemocytometer

Viable and non-viable cells were counted, using the Neubauer's hemocytometer. 4% TB was added to re-suspended TM4 cell in a ratio of 1:1: 20 µl of suspended TM4 cells was added to 20 µl of TB. 10 µl of the TB cell suspension was added to the respective chambers of the hemocytometer and was observed under an inverted

phase-contrast microscope (Zeiss). The cellular proliferation was determined by counting the total number of cells for each 24 hrs period. The percentage cell viability and cell toxicity was determined using the equations 1 and 2.

2.4 XTT mitochondrial activity cells assay

2.4.1 Assay principle

The study focused on monitoring the mitochondrial activity (MA) of the TM4 Sertoli cells. The XTT assay was used to determine the mitochondrial activity, as the XTT is converted to a formazan dye only by metabolic active cells, which corresponded to the mitochondrial dehydrogenase (MDH) activity (Smith & Hunter, 2008).

The cells were incubated with XTT reagent for 4 hrs at 37°C and 5% CO_2, resulting in the formation of formazan crystals which was quantified by determining the absorbance readings at a wavelength of 450nm, using a 96-well microtiter plate reader (GloMax®-Multi Detection System, Promega).

The XTT reagent was prepared according to the manufacturer protocol, as follows: 50 µl of yellow XTT solution was added to each well making up a final volume of 150 µl/well. Formazan formation was determining by measuring the absorbance that was read at a wavelength of between 450-600 nm. The % of MA was determined using the following equation:

$$\% \text{ MA} = \frac{\text{Experimental absorbance abs} - \text{Media abs}}{\text{Control abs} - \text{Media abs}} \times 100$$

2.4.2 Treatment with Meth for XTT assay

The TM4 cells were seeded at a density of 10^4 cells/well (n=5; day=0) in 100µl of supplemented DMEM in a 96-well TC microtiter plate (SPL, Korea), and were allowed to overnight (24 hr). After 24 hrs, the culture media (supplemented DMEM) was removed and the cells were treated with selected concentrations of

0.1, 1, 10, 20 and 100 µM for both acute (24 hrs) and chronic (treating daily for 96 hrs) treatments.

2.5 Trans-endothelial electrical resistance (TEER) across a TM4 cells monolayer

2.5.1 TEER principle

The BTB is crucial for the integrity of the germinal epithelium and has an important role in preserving spermatogenesis. The bioelectrical resistance across the TM4 monolayer was used to monitor changes in trans-endothelial permeability. Changes in resistances are specifically correlated with permeability and is thought to specifically reflect changes in the paracellular patway, which in turn, could indicate whether TJs were compromised or not (Gye, 2003). Growing TM4 cells on the mixed cellulose esters membrane (transwell filter insert) emulates the *in vivo* basement membrane of the BTB using TM4 cells to morphologically and functionally form the apical and basolateral compartments which simulates the luminal (seminiferous tubule lumen) and abluminal (blood) compartments which exist inside the *in vivo* testis microenvironment encompassing the BTB.

Across the *in vitro* blood-testis-barrier, trans-endothelial electrical resistance (TEER) was measured using an Ohm Millicell-ERS (Electrical Resistance System) (Millipore, MERS 000 01), which measures the resistance across monolayers of epithelial cells in culture. TEER is utilized to assess the integrity and permeability of epithelial monolayers, as it is an indirect measurement of the status of paracellular permeability (Derk et al., 2015).

2.5.2 Experimental treatments

The TM4 Sertoli cells were seeded on mixed cellulose esters culture inserts (Millicell®, Cat no. PIHA012) in a 24-well TC microtiter plate (SPL, Korea), at a cell density of $5x10^4$ cells/well/insert, and cultured at 37ºC and 5% CO_2 (n=3; day=0). The total volume of media in each well was 800µl (containing 300µl inside the insert, which are where the cells are seeded and 500 µl in the well, which contained the Meth). The TM4 cells were allowed to attach to the bottom of the insert overnight

and divided to confluence. The effects of Meth treatment were bio-electrically investigated for 24, 48, 72 and 96 hrs, by recording TEER readings for both treatments (acute and chronic). Before measuring TEER, each of the 24 well plates were removed from the 37ºC incubator and were allowed to acclimatize to ambient temperatures for 20-30 min in a laminar flow cabinet. Aseptic strategies were utilized to maintain culture sterility. The electrodes were sterilized by placing in EtOH for 15 min, and followed by immersing the electrodes in supplemented media for 15 min, which served as an electrolyte solution used for the stabilization of the electrode interface. For the control, TM4 cells were exposed to supplemented media only. In addition, blank inserts, which contained just a supplemented media with no TM4 cells, were used to subtract the electrical resistance of cellulose membrane (blank reading). TEER measurement was determined by measuring the resistance of the TM4 monolayer by holding the electrode until the meter indicated a stable resistance reading (experimental reading).

TEER was calculated by the following equation:

True resistance = (Experimental Reading - Blank reading) × 0.6 cm^2

*Note: Media was replaced every morning for 24-96 hours (4 days) after TEER measurements were recorded to prevent the influence of PH changes in the media to affect resistance readings across the TM4 cell monolayer.

CHAPTER 3

Results

3.1 Viability study

The effect of Meth on viability was assessed to determine whether the selected Meth concentration range used in the study had any toxic effects.

3.1.1 Effect of acute Meth exposure on cell viability of TM4 cells

Figure 3.1 represents the viability percentage of the cell population after acute Meth exposure, cells were exposed to Meth for 24 hrs and thereafter, to fresh media on a daily basis.

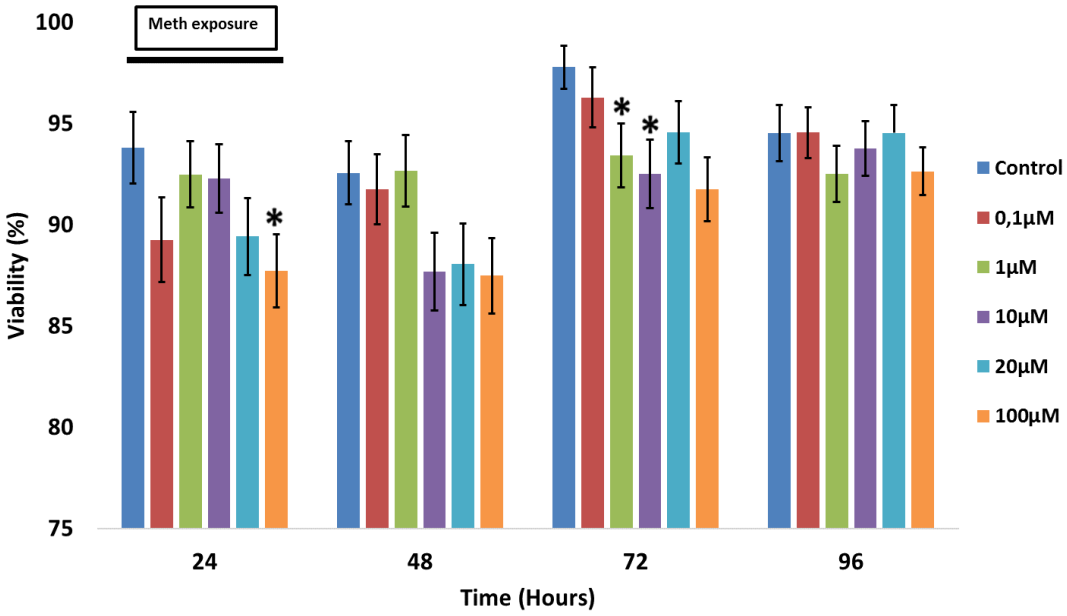


Figure 3. 1: The effect of acute Meth exposure on the viability% of TM4 Sertoli cells over the experimental period of 24-96 hrs using the Trypan Blue exclusion assay. Results were displayed as mean ± SEM (n=3). The * denotes statistical significance (P<0.05) between experimental samples as compared to controls by using *ANOVA* (Microsoft Excel 2016).

At 24 hrs, no statistical significant difference was observed in viability except at 100 µM where there was a significant decrease in viability compared to the controls (P=0.04). At 48 hrs, no statistical significant differences were observed across all the concentrations of Meth compared to the control, however, significant decrease

was observed at 72 hrs with 1 µM and 10 µM of Meth concentration, the viability% decreased compared to the control (P=0.08), whereas at 96 hrs no statistical significant differences were found when compared to the control.

3.1.2 Effect of chronic Meth exposure on cell viability

Figure 3.2 represents the percentage viability of the cell population after chronic Meth exposure.

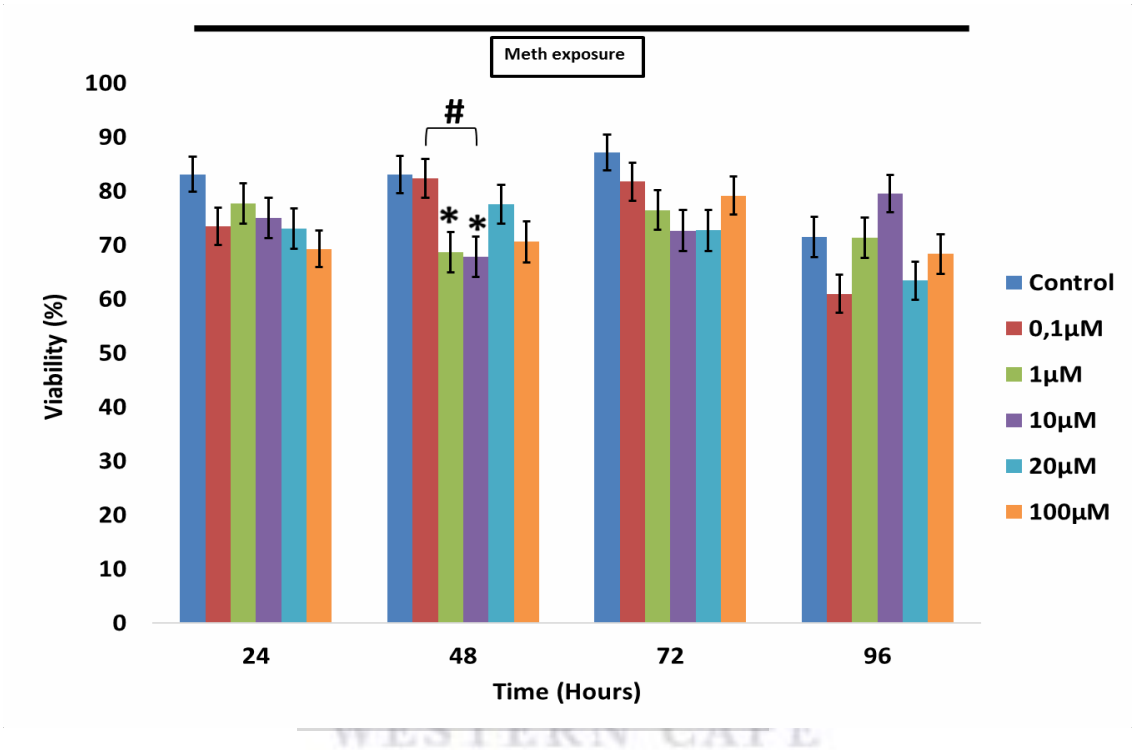


Figure 3. 2: The chronic effect of Meth exposure on the viability% of TM4 Sertoli cells over the experimental period of 24-96 hrs using the Trypan Blue exclusion assay. Results were displayed as mean ± SEM (n=3). The * denotes statistical significance (P<0.05) between experimental samples, compared to controls, # denoted statistical significant differences between experimental samples by using *ANOVA* (Microsoft Excel 2016).

The chronic exposure of TM4 to Meth for 24 hrs showed no statistical significance difference in viability% at all concentration in comparison to the control. At 48 hrs exposure, the live cell count was significantly reduced at 1 µM and 10 µM of Meth concentrations compared to the controls (P<0.008), furthermore, significant differences were obtained between 0.1 µM and 1 µM (P=0.014), also between 0.1 and 10 µM. By 72 and 96 hrs exposure, all Meth concentrations showed no significant differences in cell viability between the control and treated groups.

3.1.3 The average proliferation of TM4 Sertoli cells after acute exposure to Meth

Proliferation can be defined as the rate of cell division. Cell proliferation was calculated as the number of additional cell divisions per 24 hr period.

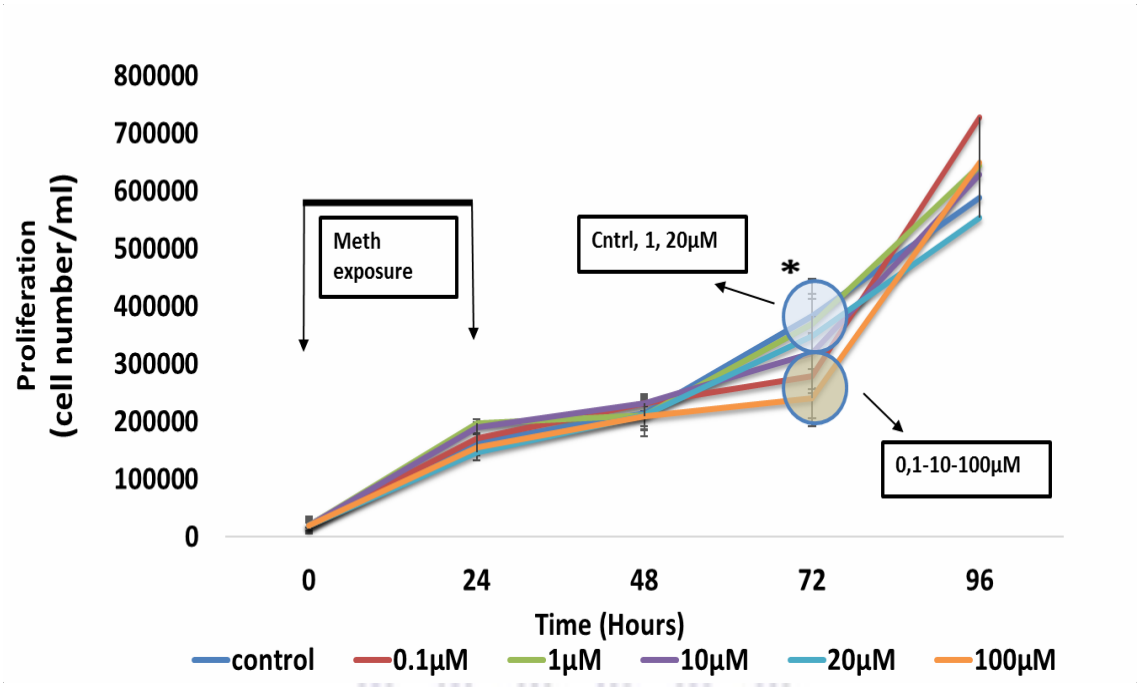


Figure 3. 3: The effect of acute 24 hrs Meth exposure on the proliferation of TM4 cells over the 96 hr experimental period using the Trypan Blue exclusion assay. Results were displayed as mean ± SEM (n=3). The * denotes statistical significance ($P<0.05$) between experimental samples, compared to controls by using *ANOVA* (Microsoft Excel 2016).

The proliferation is meant to represent the rate of Sertoli cells division over 96 hrs and indicated the number of cell divisions over the experimental time frame. Sertoli cells were acutely exposed to Meth (24 hrs) in order to determine whether Meth affected the division TM4 cell. Over the 24-96-hrs experimental time frame, no significant differences showed at 24 hrs and 48 hrs, between the control and treated groups. However, at 72 hrs Meth treatment caused a significant suppression on TM4 Sertoli cell division between the following groups [cntrl, 0.1 µM and 1 µM] and [10 µM, 20 µM and 100 µM] ($P=0.05$). No statistical significant differences were observed at 96 hrs.

3.1.4 The average proliferation of TM4 Sertoli cells after chronic exposure to Meth

TM4 cells were treated chronically with selected concentrations of Meth. Treating TM4 cells with Meth did not statistically affect the proliferation between 24 hrs and 48 hrs. (see fig 3.4)

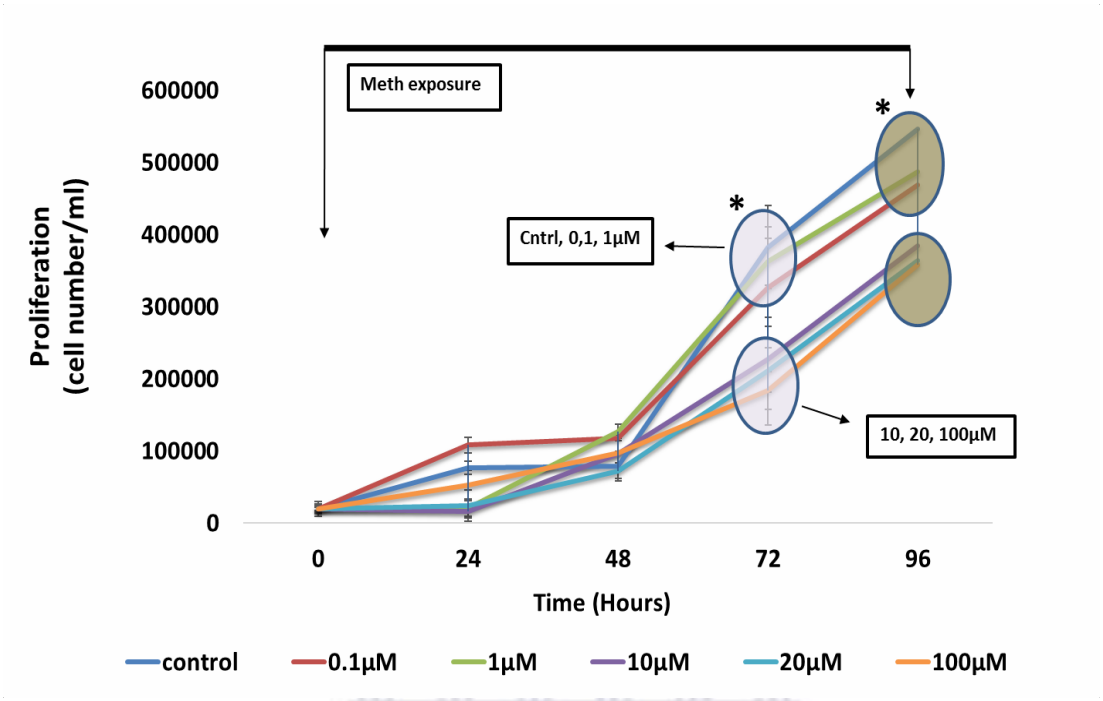


Figure 3. 4: The effect of chronic Meth exposure on the proliferation of TM4 cells over the 24-96 hrs experimental period using the Trypan Blue exclusion assay. Results were displayed as mean ± SEM (n=3). The * denotes statistical significance (P<0.05) between experimental samples as compared to controls by using *ANOVA* (Microsoft Excel 2016).

However, at 72 hrs (P<0.05) and 96 hrs (P=0.01), control, 0.1 µM and 1 µM groups were significantly different from the groups 10 µM, 20 µM and 100 µM. thus, chronic Meth exposure suppressed cell proliferation at 10 µM to 100 µM.

3.2 Mitochondria activity (MA) using the XTT assay

Tetrazolium salts are widely used to investigate cytotoxicity, cell proliferation and viability by determining the MA in cells (Smith & Hunter, 2008). We used 2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl) 2*H*-tetrazolium-5-carboxanilide (XTT), to evaluate the levels of mitochondrial dehydrogenase as an indicator of mitochondrial activity.

3.2.1 The effect of acute Meth exposure on mitochondria activity of TM4 cells

Figure 3.5 represents the acute effects of selected concentrations of Meth on the mitochondria activity (MA) using the XTT assay.

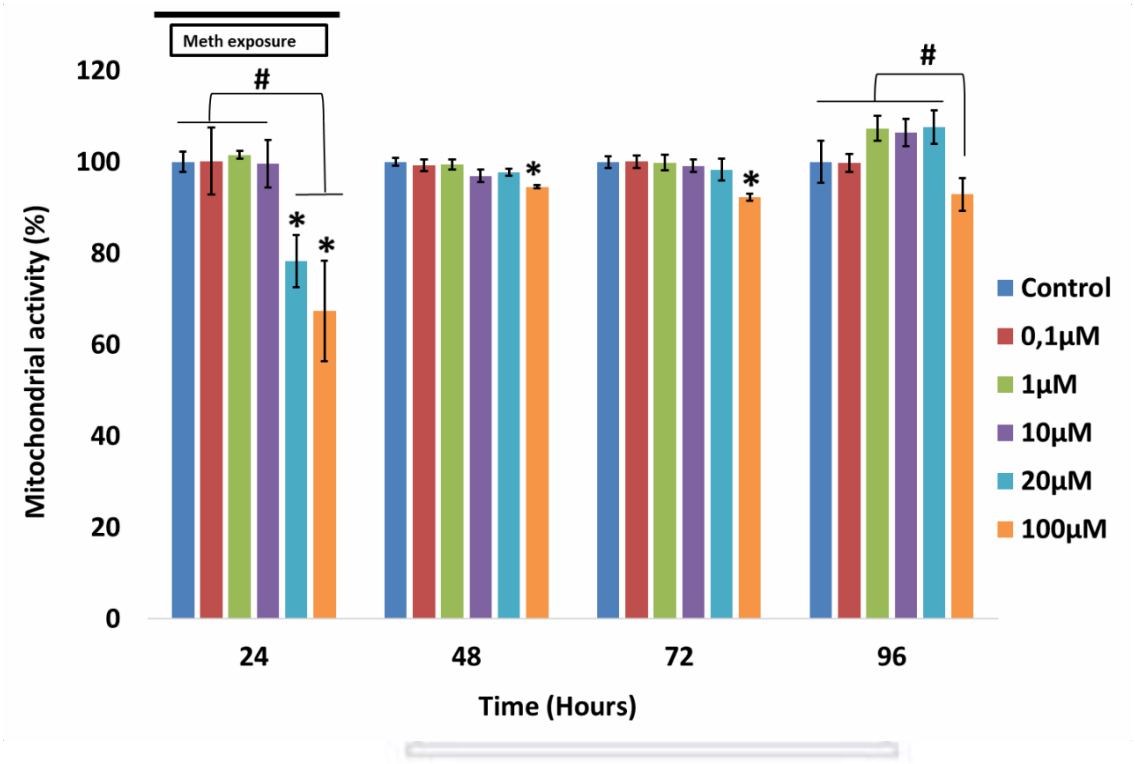


Figure 3. 5: The effect of acute Meth exposure (24 hrs) on the mitochondrial activity (%) of the TM4 Sertoli cell in comparison to controls (not exposed to Meth) at selected time intervals. The * denotes the statically significant differences between experimental samples compared to the control at ($P<0.05$), the # denotes the significant differences between the treatment groups ($P=0.05$). Data was represented as mean ± SEM (n=5) by using *ANOVA* (Microsoft Excel 2016).

The acute effect of Meth concentrations showed that at 24 hrs, there was a significant decrease in MA between control cells and 20 µM and 100 µM Meth treated cells. Comparing MA at both 20 µM and 100 µM of Meth with 0.1 µM, 1 µM and 10 µM showed a statistical significant decrease ($P<0.04$) in MA. At 48 and 72 hrs, the effects of Meth decreased MA at 100 µM compared to control group ($P=0.004$). Finally, at 96 hrs, there was an observed a significant decrease of MA at 100 µM ($P=0.0114$) when compared to the 1 µM, 10 µM and 20 µM samples groups. (see fig 3.5), but not with controls.

3.2.2 The effect of chronic Meth exposure on mitochondria activity of TM4 cells

Figure 3.6 represents the chronic effects of selected concentrations of Meth on mitochondrial activity % using the XTT assay.

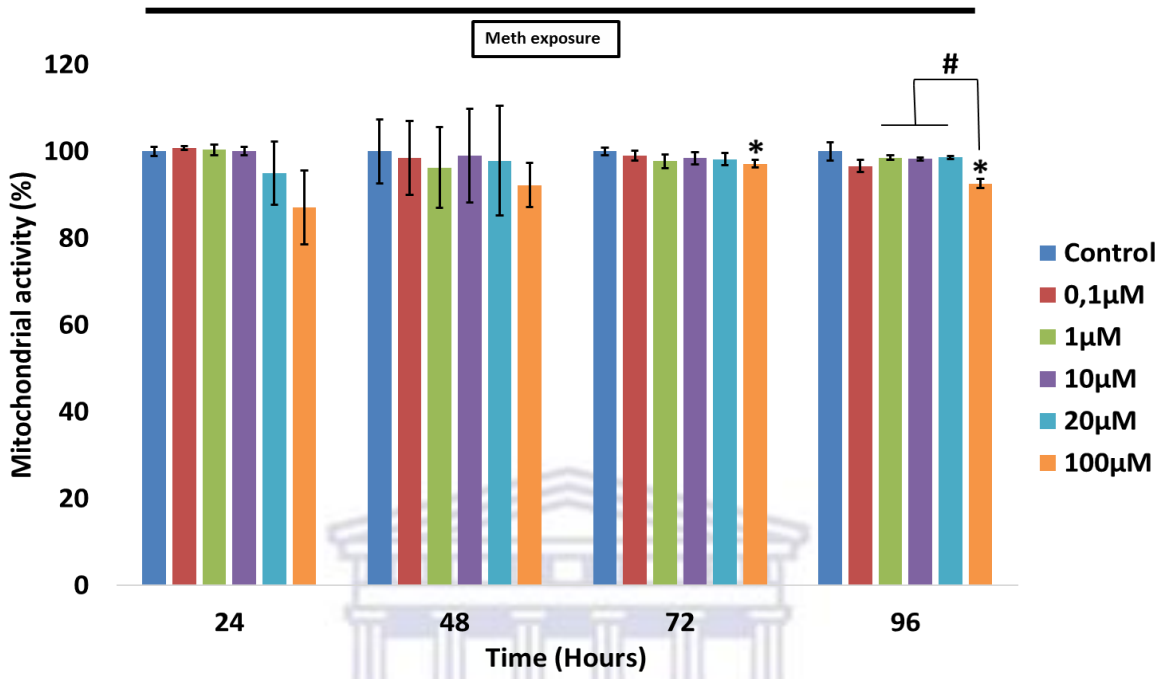


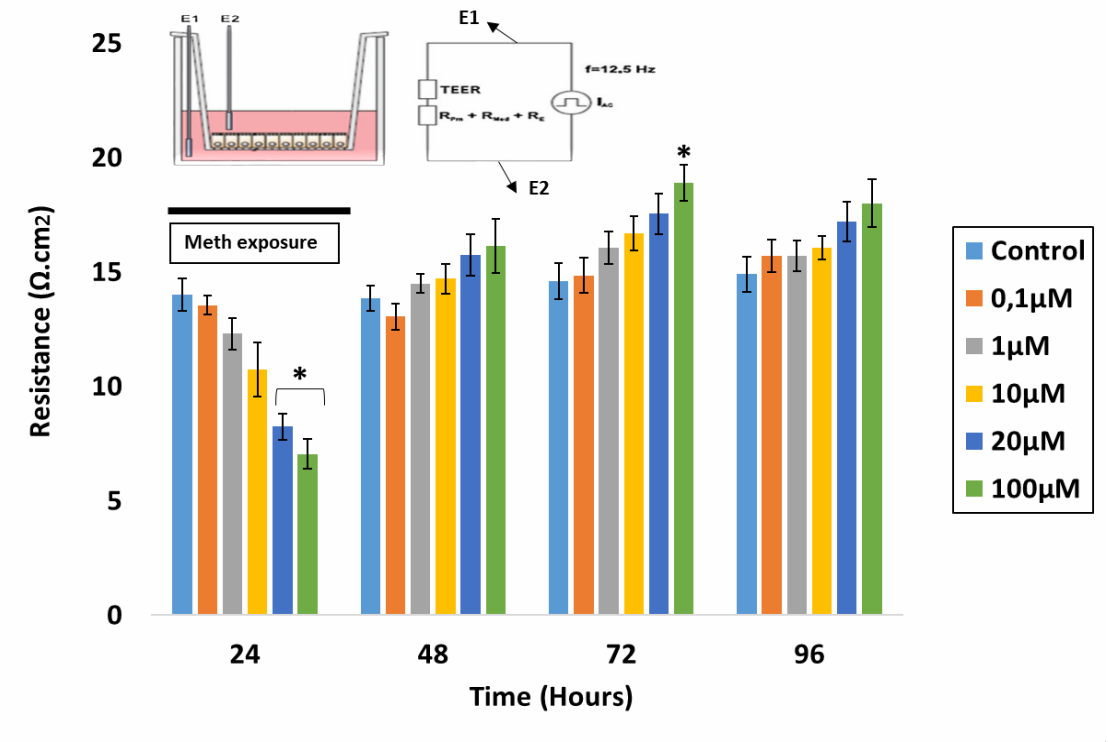
Figure 3. 6: The effect of chronic selected concentrations of Meth on the mitochondrial activity (%) of TM4 Sertoli cells over the experimental time period of 24-96 hrs. The * denotes the statically significant differences between experimental samples compared to the control at ($P<0.05$), the # denotes the significant differences between the treatment groups ($P=0.05$). Results were displayed as mean ± SEM (n=5) by using *ANOVA* (Microsoft Excel 2016).

The effect of chronic Meth exposure resulted in no statistical difference of cell proliferation at 24 and 48 hrs, however, at 72 and 96 hrs Sertoli cells showed a slight suppression when compared the control at the 100 µM Meth treated cells ($P=0.05$). Further, at 96 hrs, the data shows a statically significant difference between the control and 100 µM group ($P=0.05$), and also when compared to the sample treatment groups (1 µM, 10 µM and 20 µM) Meth ($P<0.05$).

3.3 Trans-endothelial electrical resistance (TEER)

3.3.1 The effect of acute Meth exposure on TEER across TM4 cell monolayers resistance

Figure 3. 7: The effect of acute Meth exposure on TEER across TM4 Sertoli cell monolayers compared to controls



at selective time intervals. Data are presented as mean ± SEM (n=3). The * denoted statistically significant differences in the TEER over time between experimental samples compared to the controls. Statistical significance was designated at P-value <0.05 by using *ANOVA* (Microsoft Excel 2016).

In the acute study, the samples were treated with Meth for 24 hrs. The resistance of TM4 cell monolayer decreased in a dose-related manner, however, with significant decreases observed only at 20 µM and 100 µM groups (P=0.0002). At 48 hrs no statistical difference was observed, but the dose effect was reversed, this trend continued at 72 hrs, with a non-statistical increase in TEER for increase in Meth dose, except at the 100 µM compared to the control (P=0.01). Although the trend continued at 96 hrs, no significance difference relative to the control was observed across all the experimental samples.

3.3.2 The effect of chronic Meth exposure on TM4 cells trans-endothelial electrical resistance

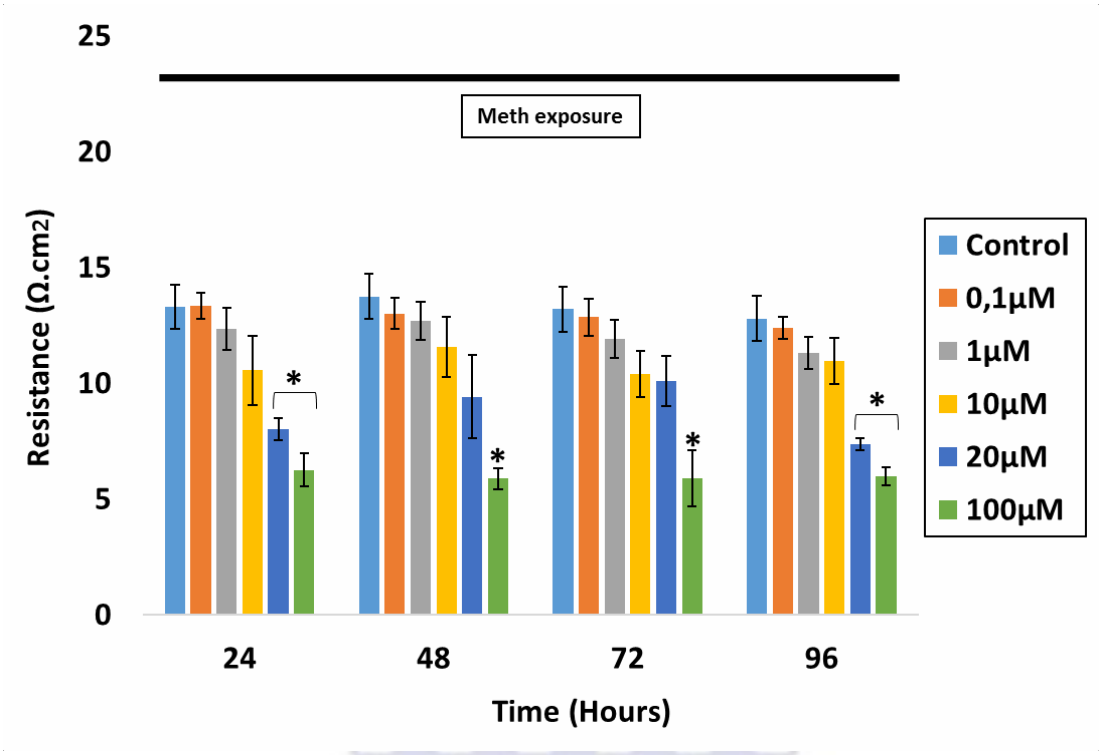


Figure 3. 8: The effect of chronic Meth exposure on TEER across TM4 Sertoli cell monolayers compared to controls at selective time intervals. Data are presented as mean ± SEM (n=3). The * denoted statistically significant differences in the TEER over time between experimental samples, relative to the controls. Statistical significance was designated at a P-value <0.05 by using *ANOVA* (Microsoft Excel 2016).

At 24 hrs a significant dose-related decrease was observed in samples that was treated with Meth (20 µM and 100 µM) when compared to the control group at (P=0.0002). At 48 and 72 hrs the trend continued with a significant decrease only when compared the control at 100 µM (P=0.024). At 96 hrs the resistance of the TM4 cells monolayer showed a significant decrease when compared the control at both 20 µM (P=0.027) and 100 µM (P=0.001) compared to the control. Thus, chronic (daily) exposure to Meth resulted in a dose-related suppression of TEER throughout the experimental time frame of 96 hrs.

CHAPTER 4

Discussion and conclusion

4.1 Introduction

The blood-testis barrier (BTB) divides the seminiferous epithelium into two compartments, basal and adluminal compartments, the latter being a specialised environment (inside the BTB) which is essential for spermatogenesis. The basal compartment is the anatomical space for spermatogonia and preleptotene spermatocytes in which to reside (Cheng & Mruk, 2012), and is considered outside the BTB.

Further development of the germ cells, primary and secondary spermatocytes, round spermatids, and elongated spermatids take place in the specialised environment of the adluminal compartment. Thus, the BTB is critical for spermatogenesis. The BTB also comprises of the germinal epithelium which is made up of two cell lines, the germ cells and the Sertoli cells. The Sertoli cells are essential for the process of spermatogenesis and is also responsible for the regulating of BTB permeability as well as maintaining the specialised environment essential for the process of spermatogenesis. Thus, studying Sertoli cells will give insight to the processes of both the maintenance of the BTB, and also the process of sperm formation.

The Sertoli cells are the only cells in the body that are stimulated by FSH which is secreted from the anterior pituitary. The TM4 Sertoli cell line used in this study, was immortalized from Balb/c mice and is used extensively for *in vitro* studies (Zhu et al., 2004; Kaitu'u-Lino et al., 2007; Ogunbayo et al., 2008; Ge et al., 2014). These TM4 Sertoli cells have FSH receptors on their plasma membrane which respond to FSH stimulation by increase cAMP formation and secreting inhibin (Tindall et al., 1981). Sertoli cells are, therefore, pertinent to investigating the effects of Meth on the BTB.

Recreational users expose themselves to Meth occasionally. Our study investigated if occasional use (exposure for 24 hrs) would have any short or long term effects on the Sertoli cells of the BTB. This type of drug use is distinctly different from

individuals who are addicted and require daily exposure (fix) to Meth. To study this type of exposure to Meth, we exposed Sertoli cells to Meth on a daily basis and this formed the basis of the chronic study.

Therefore, this study aimed to determine the effects of acute and chronic Meth exposure on TM4 Sertoli cells by investigating the viability, proliferation, mitochondrial activity and permeability of TM4 cell monolayers over a period 24-96 hrs.

4.2 Effect of acute and chronic Meth exposure on TM4 cell viability

The range of control viability of TM4 Sertoli cells was found to be between (71 % to 99 %) across the experimental time-frame. Thus, essentially viability of all Meth exposed cells remained within this normal range throughout this study. Although not statistically significant, Meth tended to slightly suppressed viability relative to controls, however, this suppression was well within the range of normality. Thus acute exposure to Meth concentration used in this study had no effect on the viability of TM4 Sertoli cells.

During chronic exposure, Meth effects on TM4 Sertoli cells were statistically significant at only 48 hrs at the concentration of 1 μM and 10 μM, where Sertoli cell viability was slightly decreased. This slight suppression also falls within the normal range of control values for viability. Thus, chronic exposure to Meth essentially did not affect TM4 cell viability in this study.

This was similar to an acute study by Zhang et al (2009) who, using human brain microvascular endothelial cells (hBMVECs) for 24 hrs, found no effects to cell viability at Meth concentrations of 100 μM, 500 μM and 1000 μM. Only at supra-physiological concentration of Meth (2500 μM, 5000 μM and 10000 μM) was cell viability significantly decreased, by eliciting a dose-related suppression of viability. In addition to this study, Martins et al. (2010) using rat brain vascular endothelial (GPNT) cells, observed no cell toxicity when exposed to 0.1 μM, 1 μM, 10 μM, 30 μM and 100 μM of Meth. Both these studies support our study regarding the effect of Meth on Sertoli cell viability (Zhang et al., 2009; Martins et al., 2010).

Furthermore, in a chronic exposure study by Fisher et al. (2015) the concentration range of Meth (0 –1,000 μM) on brain endothelial cells (bEnd5) had no significant

effect on cell viability even with the higher concentrations (supra-physiological). Thus, this study also endorses our finding for chronic Meth exposure on TM4 Sertoli cells.

In conclusion, both acute and chronic exposure of Meth at concentration of 0.1 μM - 100 μM, was not toxic to Sertoli cells.

4.3 The average proliferation of TM4 Sertoli cells during acute and chronic exposure to Meth

Proliferation is a measure of the rate of cell division while in culture. In the acute study, proliferation of Meth exposed TM4 Sertoli cells were not statistically different from controls between 24 to 48 hrs. However, at 72 hrs, 0.1 μM, 1 μM and 100 μM exhibited a suppressed proliferation compared to the control, 1 μM and 20 μM expose groups. By 96 hrs, there was no statistical difference between the rates of proliferation, which indicated that Meth tends to suppress proliferation only at 72 hrs (48 hrs) after the withdrawal of Meth. Thus, acute exposure to Meth (24 hrs) appears to have a long-term effect on Sertoli cell division (see at 72 hrs). However, the TM4 cells recovered to normal control levels by 96 hrs (see fig 3.3).

During chronic Meth exposure, proliferation between 24 to 48 hrs, was not statistically different from controls. However, at both 72 and 96 hrs, 10 μM, 20 μM and 100 μM Meth exposed Sertoli TM4 cells were suppressed relative to the control, 0.1 μM and 1 μM groups of cells. At both 72 and 96 hrs, control TM4 Sertoli cells did not statistically differ from 0.1 μM and 1 μM groups of Meth exposed TM4 Sertoli cells. The suppressed proliferation of the chronically exposed TM4 Sertoli cells indicated that daily exposure to Meth results in a persistence of the suppression at only the higher Meth concentration groups of 10 μM, 20 μM and 100 μM.

Melega et al. (2007) showed that most Meth users had a plasma concentration of 11.1 μM of Meth in their blood. It is significant that the effects of Meth on the physiology of Sertoli cell division was also only affected at levels of 10 μM and greater, the same concentration of Meth that psychologically affects and satisfies Meth addicts. This suggests a mechanism for Meth-induced infertility in male Meth users which requires of plasma concentration of 10 μM or greater to cause a

suppression in Sertoli cell division, that would also negatively affect spermatogenesis. The amount of sperm produced is proportional to the number of Sertoli cells (Hess et al., 1993; Schulz et al., 2012) in the germinal epithelium. Thus, a suppression of Sertoli cells division would result in a decrease sperm count, which in turn would substantially decrease male fertility. To date, there is no studies in the literature that report data which suggests a mechanism for Meth-induced male infertility.

This study indicates that even a short exposure to Meth has long-term effects on the cell division of Sertoli cells, but, the data also inducted that cells can recover from these acute effects by 96 hrs. Males with subfertility (decrease sperm count and decrease sperm motility) may be advised that even the occasional use of Meth may further decrease their fertility.

4.4 The effect of acute and chronic Meth exposure on mitochondrial activity of TM4 cells

Mitochondrial activity (MA) was acutely suppressed at 24 hrs at Meth concentration of 20 μM and 100 μM. However, 0.1 μM and 10 μM Meth exposure had no statistical effect on MA. It is also significant that exposure to high concentrations of Meth (100 μM) had a long-term effect, suppressing MA for up to 96 hrs.

The chronic Meth study on MA endorsed the trends seen in the acute study. However, the TM4 Sertoli cells seem to be able to adapt to continue Meth exposure at the concentration of 0.1 μM to 20 μM, where TM4 cells were statistically not different to controls throughout the experimental time-frame of 96 hrs. Once again a slight suppression of MA was seen at 100 μM Meth exposure ($P<0.05$).

MA is a measure of mitochondrial capacity and its ability to generate ATP. The relationship between MA and ATP is an indication of the Sertoli cell viability to carry out its physiological functions, namely, facilitating spermatogenesis and maintaining the integrity of the BTB. It is further clear that high doses of Meth impair the ability of the Sertoli cell to generate viable sperm. This may contribute to the mechanism of Meth-induced male infertility.

A study by Ajjimaporn et al (2005) investigated the effects of chronic Meth on dopaminergic cells (MN9D and SK-N-SH) cells, respectively, and showed that a dose-dependent decrease in cell viability which also decrease a MA as well, and that was linked to exposure of 10 μM, 100 μM, 500 μM and 1000 μM of Meth at 24 hrs. Also when the time-frame of 24 hrs was extended, exposure of 1000 μM of Meth resulted in a 32% viability status. The study mentioned the additional findings which indicated that various cell lines, such as epithelial, endothelial, neural and fibroblast-like cells, require a higher Meth concentration range to physiologically impact cell viability and MA *in vitro*.

The robust nature of the BTB to toxicity is expected in view of the need for the developing germ cells requiring protection against toxic blood-bourn substances while developing into spermatozoa (Zhang et al., 2009).

4.5 The Effect of acute and chronic Meth exposure on TM4 cells trans-endothelial electrical

Adjacent TM4 Sertoli cells have the potential to be able to grow monolayers which express tight junctions, which inturn allows for the study of the *in vitro* BTB model (Gye, 2003). It is possible to monitor the integrity of TM4 Sertoli cell monolayer by quantifying the *in vitro* trans-epithelial electrical resistance (TEER) (Benson et al., 2013). Measuring the electrical resistance across Sertoli cells monolayers (TEER) is an assessment of the permeability function of the *in vitro* blood-testis-barrier model (Carette et al., 2013). It is important to note that a decrease in TEER represents an increase in permeability across the monolayer and *vice versa*.

Acute exposure of Meth to TM4 Sertoli cell caused a dose-related decrease in TEER, with 20 μM and 100 μM of Meth showing a statistical significant at 24 hrs. After removal of Meth at 24 hrs, TEER show a reversed trend in presenting a dose-related elevation TEER between 48 and 96 hrs. This trend persisted between the 48 hrs to 96 hr time-frame of the experiment (see fig 3.7).

Daily Meth exposure resulted in the continued dose-related suppression of TEER throughout the 96 hrs experimental time-frame with 20 μM and 100 μM Meth exposure causing a significant ($P<0.05$) suppression. Whereas in the acute study the TEER response was reversed after the removal of Meth, the continued exposure of

Meth prevented this reversal of the TEER response. It is clear that for Meth to induce a decrease in TEER, it must be present (see fig 3.8) and subsequent withdrawal causes a long term alteration in the permeability status of the BTB.

The decline of resistance across the Sertoli cell monolayers in this study might be due to Meth affecting the paracellular TJs, in fact, increasing permeability in both a dose and time-dependent manner. Ramirez et al. (2009) noted that a dose-dependent decrease of 20-50% of TEER after hBMVECs were exposed to 50 μM and 250 μM Meth for 21 hrs, as a result of the partial loss of monolayer paracellular integrity.

Also, a study by Mahajan and colleagues (2008) which investigate the effect of Meth for longer than 24 hrs (10, 25 and 50 nM) using BMVECs, reported a dose-dependent decrease in TEER which was observed at 24, 48 and 72 hrs. The greater effect was observed when endothelial cells were exposed to 50 nM at 48 hrs post Meth exposure.

Our data suggests that Meth has the ability to affect the permeability across the BTB by impacting transport properties and the paracellular permeability. These effects persist over 96 hrs, suggesting long-term effects for occasional Meth exposure, on Sertoli cells and their barrier properties by changing the permeability properties across the BTB. This may induce dire consequences on the special luminal environment and its ionic makeup of the seminiferous tubules. A study by Wong and Cheng (2011) has shown that if the BTB is perturbed, meiosis is interrupted and spermatogenesis halted. Thus, this maybe another factor responsible for Meth-induced male infertility.

4.6 Future work

Firstly, investigating the mechanism for the Meth induced decrease in cell number, where cell cycle analysis may indicate where in the mitotic cell cycle does Meth suppress cell division.

Secondly, ATP studies may indicate if the suppression of cell number is due to decrease oxidative phosphorylation.

Thirdly, PCR studies may indicate whether the Meth induced decrease in permeability is based on paracellular or transcellular permeability by investigating the relative expression of the TJ proteins occludins and claudins after exposure to Meth.

Last, *in vivo* studies are crucial to investigate these findings in an animal model to evaluate sperm motility and morphology parameters, which would also provide useful insights into the effects of Meth induced infertility in males.

CHAPTER 5

5.1 References

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5.2 Web-based References

Figure 1.1 (Chapter 1) (http://www.atlantaduilawyer.com/possession-of methamphetamine -georgia/) [Accessed 24 June 2016].

Figure 1.4 (Chapter 1) (https://nursekey.com/reproductive-system-3/) [Accessed 3 April 2017].

APPENDIX

Appendix A

Cell freezing protocol

After cells were reached to 80 % confluence prior to cell freezing, therefore, to remove compounds that might interact with the action of trypsin, cells rinsed with 3 ml sterile PBS and the growth medium was discarded. 2 ml of 0.25 trypsin was added allowed to cover the flask surface and incubated at 37°C until cells began to detach. Normally, this took approximately 5 to 6 minutes.

Once TM4 cells detach, 2 ml of supplemented growth medium was added to stop the effect of trypsin, cells were re-suspended by repeated aspiration. TM4 were counted before freeze. The detached cells were transferred to a 15 ml conical tube and centrifuge at 2500 rpm for 6 minutes. The supernatant was removed thereafter, the cell pellet was re-suspended in 10 ml freezing medium (87.5 % DMEM/ F-12, 15 % HS, 2.5% FBS and 5 % DMSO), aliquot into cryogenic vials and immediately taken to -80 °C for 24 hrs, thereafter, transferred the vials to the liquid nitrogen for long-term storage.

Appendix B

Cell thawing protocol

After cells were taken from the liquid nitrogen, the vials containing the frozen TM4 cells were placed into water bath heated to 37 °C until the crystal been dissolved. Once the crystal has disappeared the TM4 cells were transferred to a 15 ml conical tube containing 10 ml of DMEM/ F-12 supplemented medium, then the suspension was centrifuged at 2500 rpm for 5 min the supernatant was removed under sterile condition and the pellet was re-suspended in 10 ml fresh DMEM/ F-12 medium, the cells then were transferred to a 75 cm^2 flask and left serene in the incubator for 48 hrs.