The implementation of *in vitro* assays to screen environmental samples for male reproductive toxicity

By

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Declaration

I, Mozaffar Ebrahim declare that the thesis entitled 'The implementation of *in vitro* assays to screen environmental samples for male reproductive toxicity' is my work and has not been submitted for any degree or examination at any other university and that all sources of my information have been quoted as indicated in the text and/or list of reference.

Full name: _____ Date: _____

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Appendix 1: Ebrahim M, Pool EJ (2010). The effect of *Tulbaghia violacea* extracts on testosterone secretion by testicular cell cultures. Journal of Ethnopharmacology 132: 359-361.

List of abbreviations

˚C -	- Degrees celsius
µg/ml	- micrograms per milliliter
µl	- microliters
µM	- micromole
17ß-HSD	- 17ß-hydroxysteroid dehydrogenase
3ß-HSD	- 3ß-hydroxysteroid dehydrogenase
ACC	- American Chemistry Council
ANOVA	- Analysis of variance
ATSDR	- Agency for Toxic Substances and Disease Registry
BPA	- Bisphenol A
BSA	- Bovine serum albumin
cAMP	- cyclic adenosine 3',5'-cyclic monophosphate
Cd	- Cadmium
cells/ml	- cells per milliliter
CO_2	- Carbon dioxide
DDE	- 1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene
DDT	- Bis (4-chlorophenyl)-1,1,1-trichloroethane
DES	- Diethylstilbestrol
DMSO	- Dimethyl sulfoxide
EDCs	- Endocrine-disrupting compounds
EE2	- 17α-Ethinylestradiol
ELISA	- Enzyme linked immunosorbant assay
EPA	- Environmental Protection Agency

ER	- Estrogen receptor
FSH	- Follicle-stimulating hormone
g	- Grams
GnRH	- Gonadotropin-releasing hormone
HPT	- Hypothalamus, Pituitary, Testicular (HPT) Axis
KET	- Ketoconazole
LDH	- Lactate dehydrogenase
LH	- Luteinizing hormone
mg/ml	- milligrams per milliliter
ml	- milliliter
mu/ml	- milliunits per milliliter
ng/ml	- nanograms per milliliter
nm	- nanometres
OD	- Optical density
P450c17	- Cytochrome P450 17α-hydroxylase/C_{17-20} lyase
$P450_{SCC}$	- Cytochrome P450 side-chain cleavage enzyme
PCBs	- Polychlorinated biphenyls
pg/ml	- picograms per millilitre
StAR	- Steroid acute regulatory protein
TCS	- Triclosan
w/v	- weight to volume
WHO	- World Health Organization
x g	- Gravitational force
XTT	- 2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5carboxanilide

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Abstract

The implementation of *in vitro* assays to screen environmental samples for male reproductive toxicity

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Keywords: male reproductive system, testosterone, estradiol, endocrine-disrupting compounds, reproductive toxicity, *Tulbaghia violacea* Harv., *Sutherlandia frutescens*(L.) R.Br., *Artemisia afra* Jacq. Ex Willd., heavy metals

Endocrine–disrupting compounds (EDCs) are exogenous compounds/chemicals which interfere with, or have adverse effects on the production, distribution and function of natural hormones, thereby affecting normal endocrine activity, health and quality of life of both humans and wildlife. The reproductive system is highly susceptible to EDCs due to it being controlled by an array of hormonal signals. The effects of EDCs on the male reproductive system include infertility, decreased sperm count, function and morphology, abnormal development of secondary sex characteristics, reproductive function and sexual behaviour as well as decreased libido. There are various sources by which EDCs enter the environment which include effluents from several industries (mining, agriculture, smelting, hazardous waste sites, manufacturing industries, etc.), sewage treatment effluents, urban and agricultural runoff and effluents which include natural and pharmaceutical chemicals excreted in the urine of humans and domestic livestock, pesticides, polychlorinated biphenyls, dioxins,

plasticizers, surfactants, etc. Humans and animals can also be affected by EDCs by consuming food containing endocrine active substances.

The growing concern regarding adverse effects due to EDC exposure of humans and wildlife, as well as the increased incidence of EDC contamination has prompted extensive research into the development and validation of screening tests to detect and monitor known EDCs and new substances with endocrine-disrupting capability. These screening tests involve assessing the effect of known and potential EDCs on reproductive function and development as well as hormone production. To assess the effect of EDCs on the reproductive system different methods are employed which include *in vitro*, *in vivo* and *ex vivo* methods. *In vitro* methods have been suggested as a suitable screening tool for EDC monitoring due to low costs, reduced animal usage, the use of standard and basic equipment as well as the ability to screen a large number of samples with multiple endpoints. Of the available *in vitro* methods, the minced testes method has been suggested as the most suitable method for screening EDCs and for this reason has been employed in this study.

The aim of this study was thus to employ a minced testes method to screen samples for male reproductive toxicity using cell viability and hormone production (testosterone and estradiol) as endpoints.

The first objective of this study was to optimize an *in vitro* testicular cell culture assay by determining both optimal luteinizing hormone (LH) concentration and incubation time needed for testosterone production. Testicular cell cultures were prepared and cells were treated with varying concentrations of LH (10, 1, 0.1, 0.01 and 0 mu/ml) and incubated for 4 hours and 20 hours. Testosterone production was evaluated for each incubation period.

Testosterone production was significantly increased for both incubation periods at all LH concentrations tested as compared to the control. For both incubation periods, there was no significant difference in testosterone production between the different LH concentrations tested. From the data obtained, the 4 hour incubation period as well as the LH concentration of 10 mu/ml were selected as optimal for the testicular cell culture assay.

The second objective of this study was to determine the effect of *Tulbaghia violacea* Harv. on the male reproductive system. *T. violacea* is a plant species indigenous to southern Africa and is used locally as a herbal remedy/medicine to treat several ailments. Cells were treated with varying concentrations of the *T. violacea* ethanol extract (with/without LH-treatment) and incubated for 4 hours. Hormone production and cell viability were evaluated. The results obtained from this pilot in vitro study demonstrated that the ethanol extract of *T. violacea* has androgenic properties by significantly increasing LH-induced testosterone production in mouse testes with no significant change in cell viability.

The third objective of this study was to assess the effect of *Sutherlandia frutescens*(L.) R.Br and *Artemisia afra* Jacq. Ex Willd. on the male reproductive system. *S. frutescens* and *A. afra* are also plant species indigenous to southern Africa and used locally as a herbal remedy/medicine to treat several ailments. Ethanol extracts of each plant was prepared and cells were treated with varying concentrations of each extract (0, 156.25, 312.5, 625, 1250, 2500 and 5000 µg/ml) with or without LH-treatment and incubated for 4 hours. Cytotoxicity by LDH measurement and hormone production (testosterone and estradiol) were endpoints that were evaluated. The results obtained showed that the ethanol extracts of both plants are not cytotoxic to testicular cells and that *A. afra* decreases testosterone production at high concentrations.

The fourth and final objective of this study was to assess the acute effect of four heavy metals, namely manganese, copper, cadmium and magnesium on the male reproductive system. These heavy metals are used extensively in manufacturing and mining industries. Cells were treated with varying concentrations of each metal salt (200, 100, 50, 25, 12.5, and 6.25 µM) with or without LH-treatment and incubated for 4 hours. Endpoints evaluated included cell viability, testosterone and estradiol production. The results obtained showed that manganese, cadmium and copper are highly toxic to testicular cells *in vitro* and therefore may potentially cause reproductive toxicity.

CHAPTER 1

Literature review

1.1 Endocrine system

The endocrine system is made up of several glands, each secreting specific hormones (chemical messengers) which travel through the bloodstream to target tissues or cells throughout the body where they exert their action (ACC, 2000). The glands that are involved in the endocrine system include the pancreas, pituitary, thyroid and parathyroid glands, adrenal glands, thymus gland, pineal gland, ovaries in the female and testes in the male (Seeley *et al*, 2003). This complex and tightly controlled system is responsible for the control of bodily processes such as growth and development, reproduction, regulation of blood cell production as well as circulation, digestion and absorption. The endocrine system also helps maintain homeostasis within the body by regulating metabolism, water and electrolyte balance (Mader, 2001; Seeley *et al*, 2003).

When hormones reach their target tissues or cells, they bind to specific intracellular or membrane-bound receptors in a lock and key manner. Once bound to a receptor, a cascade of intracellular events are triggered which include changes in ion channel permeability, phopshorylation or dephosphorylation of cytoplasmic proteins or increased intracellular molecule concentrations (Sturmhöfel and Bartke, 1998; Seeley *et al*, 2003).

The endocrine system is so complex that a single gland can produce one or several hormones, each having one or more target cells or organs with single or multiple effects. Furthermore,

the degree of hormone response is dependent upon secretion rate and the period of time by which the hormone concentration is reduced by half (half-life) as well as the number of available target cells and cell receptors as well as the amount of binding protein in the blood (Seeley *et al*, 2003).

1.2 Endocrine disruption

Endocrine disruption can occur as a result of exposure to endocrine-disrupting compounds (EDCs). EDCs can be defined as exogenous compounds/chemicals which interfere with, or have adverse effects on the production, distribution and function of natural hormones, thereby affecting normal endocrine activity, health and quality of life of both humans and wildlife (Clotfelter *et al*, 2004). EDCs are found in all components of the environment. They can be of natural (estrogens/androgens and phytoestrogens), synthetical (Ethinylestradiol, DDT, etc.) and industrial (Nonylphenol, dioxins, etc.) origin (Ishibashi *et al*, 2001). The reproductive system is highly susceptible to these compounds/chemicals due to it being controlled by an array of hormonal signals (Mills and Chichester, 2005). There are several mechanisms by which EDCs can interfere with transportation, biosynthesis, metabolism and binding of hormones (Clotfelter *et al*, 2004), one of which is hormone mimicry. EDCs can mimic hormones by binding to estrogen or androgen receptors resulting in stimulation or inhibition of hormone synthesis. In this way endocrine disrupting chemicals can have both agonistic and antagonistic effects (SETAC, 2000). EDCs can also stimulate or inhibit the activity of enzymes, leading to faster or slower degradation or synthesis of hormones resulting in inadequate performance of intended hormone function whilst some EDCs can harm hormone-producing glands rendering them non-functional (SETAC, 2000; Clotfelter *et al*, 2004).

1.3 Male reproductive system

1.3.1 Overview

The male reproductive system is made up of the testes, vas deferens, epididymis, urethra, seminal vesicles, prostate gland, bulbourethral gland, the penis and scrotum. The maintenance and function of this intricate system is dependent upon hormonal and neural mechanisms. With regards to reproductive hormones, they are responsible for the development, maintenance and function of the reproductive system as well as the development of secondary sex characteristics, sexual behaviour and spermatogenesis (Mader, 2001; Seeley *et al*, 2003).

1.3.2 Hypothalamic-pituitary-testicular (HPT) Axis

The Hypothalamic-pituitary-testicular (HPT) axis is made up of the hypothalamus, pituitary and testes. This axis is the hormonal mechanism by which sex hormone secretion is regulated. This hormonal mechanism begins with the hypothalamus where gonadotropin-releasing hormone (GnRH) stimulates the release of both luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary gland (anterior). LH stimulates the Leydig cells, which are interstitial cells found adjacent to the seminiferous tubules within the testes, to secrete testosterone, whilst FSH stimulates Sertoli cells, which are found within the seminiferous tubules, to stimulate spermatogenesis. A negative-feedback mechanism exists where testosterone inhibits LH and FSH secretion by inhibiting the release of GnRH from the hypothalamus whereas inhibin inhibits FSH release from the anterior pituitary (Mader, 2001; Seeley *et al*, 2003).

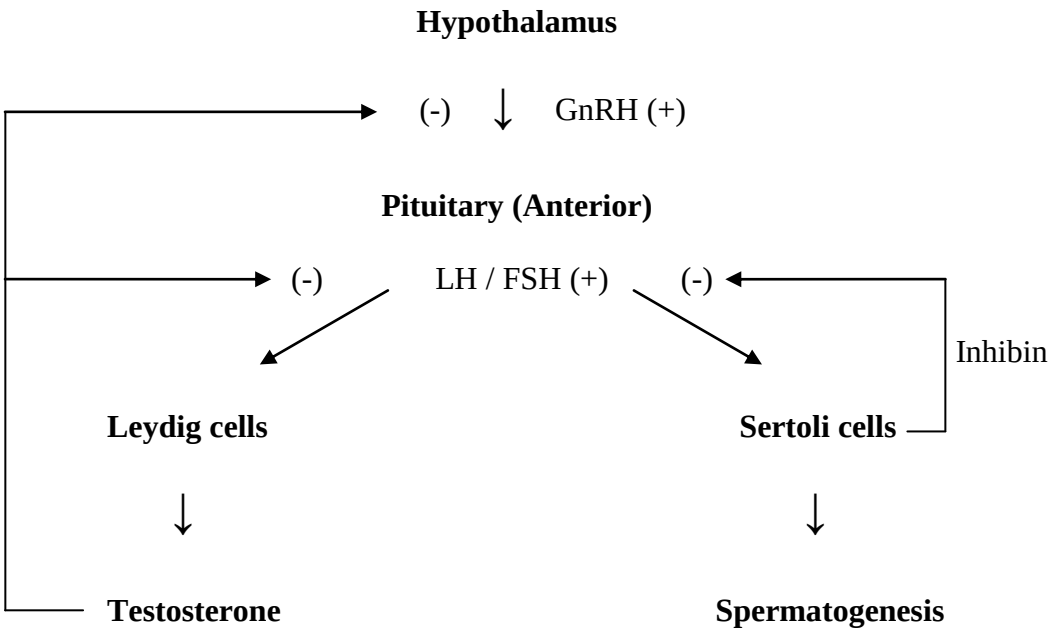

Figure 1.1 Summary of the Hypothalamic-pituitary-testicular (HPT) axis (Mader, 2001; Seeley *et al*, 2003)**.**

1.3.3 Testosterone

Testosterone is an anabolic steroid belonging to a group of endocrine hormones known as androgens and is produced in both males (testes) and females (ovary). In males, it is the main sex hormone responsible for a number of bodily processes which include the development of secondary sex characteristics, reproductive function, sexual behaviour as well as aiding in spermatogenesis (Mader, 2001; Seeley *et al*, 2003). Testosterone has been widely used as a marker of androgenicity (Walton *et al*, 1995; Kumar *et al*, 2008; Yakubu *et al*, 2008). Testosterone is also associated with muscle and bone growth and also increases muscle mass and strength (Mudali and Dobs, 2004; Yakubu *et al*, 2008). It is produced in the testes by the Leydig cells under the stimulation of LH from the pituitary gland (Gail and Hedger, 1992; Kumar *et al*, 2008).

In brief, LH binds to membrane-bound LH receptors on Leydig cells which then set off a cascade of intracellular events beginning with steroid acute regulatory protein (StAR), which transports cholesterol to the inner mitochondrial membrane to be converted by cytochrome $P450_{SCC}$ to pregnelonone (Reinhart *et al*, 1999). Pregnelonone, in turn, is converted to progesterone by 3ß-hydroxysteroid dehydrogenase (3ß-HSD). Cytochrome P450 17α-hydroxylase/C_{17-20} lyase (P450c17) then converts progesterone to 17α-hydroxyprogesterone and subsequently androstenedione (Lieberman and Warne, 2001). Testosterone is then formed by the reduction of androstenedione by 17ß-hydroxysteroid dehydrogenase (17ß-HSD) (Stocco, 2002; Hsu *et al*, 2003). Testosterone can further be converted to estradiol by aromatase, an enzyme which converts androgens to estrogens by hydroxylation and aromatization (Brodie *et al*, 2001; Carreau *et al*, 2001; Harden and MacLusky, 2004).

A deficiency in testosterone production can have major adverse effects on reproductive health as well as other bodily functions and processes. Testosterone deficiency been associated with aging (Morley and Perry III, 2000; El-Sakka and Hassoba, 2006) as well as with several diseases and disorders which include metabolic syndrome, hypogonadism, osteoporosis, obesity, diabetes mellitus type II, erectile dysfunction, Alzheimer's disease and cardiovascular disease (Jones, 2007; Schulman *et al*, 2009). Effects of low testosterone levels include decreased libido and low sperm count (Zitzmann, 2008), infertility, decreased muscle mass and strength, cognitive impairment and depression (Morley and Perry III, 2000; Zitzmann and Nieschlag, 2000; Schulman *et al*, 2009).

1.4 Reproductive toxicity

Variations in the steroidogenesis pathway can result in an inhibition or stimulation of sex hormone synthesis, resulting in a hormonal imbalance which can cause adverse effects on the reproductive system (EPA, 2005). Reproductive toxicity can occur at hypothalamic-pituitary level where toxicants can alter the function of these glands (hormone production – GnRH, LH, FSH) (Fisher, 2004), or at a testicular level where toxicants can directly disrupt hormone production (testosterone production by Leydig cells – steroidogenesis) (Murugesan *et al*, 2005; Kumar *et al*, 2008) and cause cell death (Kim and Soh, 2009, Yang *et al*, 2003).

Cholesterol (inner mitochondrial membrane)

↓ $P450_{SCC}$

Pregnelonone

↓ 3ß-HSD

Progesterone

↓ P450c17

17α-hydroxyprogesterone

↓ P450c17

Androstenedione

↓ 17ß-HSD

Testosterone

Figure 1.2 Summary of testosterone synthesis within the Leydig cells from cholesterol to testosterone (Mader, 2001; Seeley *et al*, 2003).

1.4.1 EDCs and the male reproductive system

Several compounds have been identified as EDCs, many of which are estrogenic or anti-androgenic exerting their effects either directly or indirectly on the reproductive system resulting in reproductive toxicity. EDC effects are mediated through the activation and transcription ability of estrogen (Pawlak and Wiebe, 2007) and androgen receptors (Filby *et al*, 2007). The effect of EDCs on the male reproductive system include infertility, decreased sperm count, function and morphology, abnormal development of secondary sex characteristics, reproductive function and sexual behaviour as well as decreased libido and testicular cancer (Gray Jr., 1998; Ong *et al*, 2002; Snyder, 2003).

1.4.2 Sources of EDCs and their effects

EDCs enter the environment via several industries, agriculture, sewage treatment, urban and agricultural runoff and effluents which include natural and pharmaceutical chemicals excreted in the urine of humans and domestic livestock, organochlorine pesticides, polychlorinated biphenyls, polynuclear aromatic hydrocarbons, dioxins, plasticizers and surfactants (Folmar *et al*, 2002).

1.4.2.1 Industrial Chemicals

Industrial pollution is a major source by which EDCs enter the environment. Several industries such as mining, agriculture, smelting, hazardous waste sites, manufacturing industries, etc. have been shown to be major contributors of EDC contamination (Calderón *et al*, 2003; Khan *et al*, 2008).

1.4.2.1.1 Bisphenol A (BPA)

Bisphenol A (BPA) is a plasticizer used in the manufacturing of epoxy and polystyrene resins (Kato *et al*, 2006) and polycarbonate plastics where it used in lacquer coatings in food and beverage containers and food cans. Studies have shown that BPA can leach out from these containers and cans into food and beverages and can be absorbed by the human body once consumed (Brotons *et al*, 1995; Olea *et al*, 1996; Nakamura *et al*, 2010). BPA also enters the environment through sewage effluent as well as landfill leachate (Crain *et al*, 2007). BPA exposure in male animals has been shown to result in decreased sperm count, testosterone, LH as well as decreased testes, epididymis, seminal vesicle and prostate gland weights (Akingbemi *et al*, 2004; Nakamura *et al*, 2010). BPA has also been shown to cause cell death in cultured rat Sertoli cells (Iida *et al*, 2003). Effects observed in wildlife species include alterations in sex determination and gonadal function (Crain *et al*, 2007).

1.4.2.1.2 Polychlorinated biphenyls (PCBs)

Polychlorinated biphenyls (PCBs) are synthetic chemicals used extensively in electrical equipment (Davis *et al*, 2007), as lubricants and coolants (ATSDR, 2001). PCBs have been shown to cause an inhibition in testicular androgenesis in adult rats (Andric *et al*, 2008; Murugesan *et al*, 2008). In a study where *Xenopus laevis* frogs were exposed to PCBs, the authors found that the development of secondary sex characteristics was significantly impaired resulting in demasculinization (Qin *et al*, 2007).

1.4.2.1.3 Cadmium (Cd)

Cadmium (Cd) is a heavy metal which has been shown to cause adverse effects in several organ systems which include the liver, kidneys and testes (Åkesson, 2005; Kim and Soh, 2009; Siu *et al*, 2009), causing toxicity in both humans and animals (Kirkham, 2006; Thompson and Bannigan, 2008). It has also been shown to be carcinogenic (IARC, 1993; Waalkes, 2000; Yamada *et al*, 2009) by inducing tumours in the prostate, testes and lungs (Waalkes, 2000; Zhou *et al*, 2004). Widespread in the environment, industrial Cd exposure is as a result of mining, smelting, combustion of fossil fuels (Nordberg *et al*, 1992; Zhou *et al*, 2004; Thompson and Bannigan, 2008), agricultural run-off, leechate from landfill sites (Thompson and Bannigan, 2008), battery and dye manufacturing and during refining of metals (Siu *et al*, 2009). General population exposure to Cd is associated with contaminated drinking water, food and cigarette smoke (WHO, 2000; Zhou *et al*, 2004; ATSDR, 2008; Siu *et al*, 2009).

1.4.2.2 Pesticides

Pesticides are substances used on their own or as mixtures to control, prevent or eradicate pests such as insects, arachnids, fungi, etc. They are toxic environmental contaminants which have been found in water, soil, air as well as in animal and human tissue (Anwar, 1997; Clementi *et al*, 2008). Several of these pesticides have been shown to cause adverse effects to the reproductive system of both humans and wildlife. Adverse reproductive and developmental effects such as demasculinization have been suggested to be due to the interaction of pesticides with estrogen and androgen receptors (LeBlanc *et al*, 1997).

1.4.2.2.1 Bis (4-chlorophenyl)-1,1,1-trichloroethane (DDT)

Bis (4-chlorophenyl)-1,1,1-trichloroethane) (DDT) is an organochlorine pesticide (Fox *et al*, 1998) used mostly to eliminate malaria vectors (Dalvie *et al*, 2004). Studies have found that DDT exposure resulted in decreased sperm count (Singer, 1949), stillbirths (Cocco *et al*, 2005) and it has been suggested to play a role in pancreatic cancer and neuropsychological dysfunction (Beard, 2006). DDT treatment of male Japanese quail resulted in a significant decrease in testosterone production, sexual behaviour and cloacal gland (Hallidin *et al*, 2005). The adverse effects caused as a result of DDT exposure in several animal species has been shown to be linked to its hormone-mimicking properties (Cocco *et al*, 2006). The DDT metabolite 1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene (DDE) has also been shown to cause adverse effects on the reproductive system (Kang *et al*, 2004; Adamsson *et al*, 2009).

1.4.2.2.2 Triclosan (TCS)

Triclosan (5-chloro-2-(2,4-dichlorophenoxy)-phenol) (TCS) is a synthetic antimicrobial agent used in several cosmetic products such as shampoo, toothpaste, and soap (Black and Howes, 1975). TCS is also used in other products which include toys, clothes, and plastics for food packaging as well as in domestic detergents (Geens *et al*, 2009). Rat Leydig cells treated with varying concentration of TCS, resulted in a significant, dose-dependent decrease in testosterone production as a result of down regulation of $P450_{SCC}$, 3ß-HSD, P450c17, 17ß-HSD as well as decreased expression of StAR (Kumar *et al*, 2008).

1.4.2.2.3 Prochloraz

Prochloraz is a fungicide which has been shown to cause major adverse effects to the reproductive system. Feminization occurred in treated male rats where nipple retention, reduced anogenital distance, hypospadias, phallus clefting as well as the induction of vaginal morphology was observed (Noriega *et al*, 2005). In a similar study by Laier *et al* (2006), the above mentioned effects were also observed including decreased testicular and serum testosterone concentrations.

1.4.2.3. Pharmaceuticals

A number of pharmaceuticals have been found to cause adverse effects to the reproductive system. Many of these are excreted in the urine or faeces which then contaminate the environment as sewage effluents (Folmar *et al*, 2002). Other ways in which these chemicals enter the environment include domestic disposal of medicines via sink or toilet, untreated sewage, as well as hospital and pharmaceutical production facilities effluents (Santos *et al*, 2010).

1.4.2.3.1 17α-Ethinylestradiol (EE2)

17α-Ethinylestradiol (EE2) is a pharmaceutical estrogen (Folmar *et al*, 2002) that is found in female contraceptive pills (Zuo *et al*, 2006). In a study where male rats were treated with EE2, the authors found that EE2 significantly decreased testosterone levels in serum and the testis, sperm head counts, and mass of the testis, epididymis and prostate. Male Japanese quail exposed to EE2 displayed decreased sexual behaviour (Hallidin *et al*, 2005).

Long term exposure of zebrafish to EE2 caused sex reversal, abnormal testes and non-expressible sperm in males (Nash, 2004).

1.4.2.3.2 Diethylstilbestrol (DES)

Diethylstilbestrol (DES) is a potent synthetic estrogen (Fenaux *et al*, 2004). The best documented example of endocrine disruption in humans involved *in utero* exposure to high doses of DES which was administered to pregnant woman to prevent miscarriage. Its use resulted in adverse reproductive effects in human offspring such as genital tract abnormalities, vaginal adenosis and adenocarcinoma at puberty in females as well as epididymal cysts, hypospadias, reduced sperm count and testicular hypoplasia in males (Snyder, 2003; Newbold, 2004).

1.4.2.3.3 Ketoconazole (KET)

Ketoconazole (KET) is a broad spectrum antifungal drug which has been shown to cause reproductive toxicity in both humans and wildlife (Amin, 2008). A dose-dependent decrease in serum testosterone concentrations was observed in rats (Adams *et al*, 1998) and patients (Pon, 1987) treated with KET. KET has been used to treat advanced prostate cancer due to its anti-androgenic properties (Johnson *et al*, 1988; Rodriguez and Acosta, 1995).

Other pharmaceuticals shown to cause adverse effects to the reproductive system include tetracycline (Farombi *et al*, 2008) and DA-125 (anthracycline) (Kim *et al*, 1999).

1.4.2.4 Phytoestrogens

Many plants contain phytoestrogens (McVey *et al*, 2004) which are estrogen-like chemicals/substances (Brožic *et al*, 2006; Rice and Whitehead, 2007) such as isoflavones, lignans, dihydrochalcones, and coumestans (Tempfer *et al*, 2007). Their classification as phytoestrogens is as a result of their ability to bind to estrogen receptors (ER) and thereby initiating estrogen-dependent transcription (Rice and Whitehead, 2007). Several studies have been conducted to determine the effects of phytoestrogens in wildlife and humans (Jones *et al*, 2002; Cui *et al*, 2005; Clotfelter and Rodriguez, 2006; Cong *et al*, 2006; Corbitt *et al*, 2007; Katsanou *et al*, 2007). The effect of phytoestrogens on the reproductive system is a major concern as phytoestrogens are found in several food products (McVey *et al*, 2004). Many plants have and are being used as traditional herbal remedies/medicines to treat several ailments and diseases as an alternative to pharmaceuticals (van Wyk *et al*, 1997; van Wyk and Gericke, 2000).

1.4.2.4.1 Isoflavones

Isoflavones are a sub-class of flavonoids (Cornwell *et al*, 2004). Genistein, equol and diadzein are the main isoflavones (Knight and Eden, 1995). They are present in legumes, lentils, chickpeas as well as soy products which include soy milk, soybeans, soy flour and soy-based infant formulas (McVey *et al*, 2004). Corbitt *et al* (2007) found that cloacal protuberance was significantly affected in male songbirds (*Junco hyemalis*) which were fed a soyprotein diet indicating reproductive system impairment. A study by Cong *et al* (2006) investigating the effect of the phytoestrogen quercetin on gonadal development in *Xenopus laevis* frogs showed that both feminization and impaired testicular development occurred.

Genistein, an isoflavonic compound found in soybeans and other soy products, resulted in reduced prostate weight, pituitary LH and testicular and plasma testosterone concentrations in rats. Genistein interferes with LH-receptor binding and thereby steroidogenesis (Hancock *et al*, 2009). Genistein also caused significant behavioural changes in the fish species *Betta splendens* (Clotfelter and Rodriguez, 2006).

1.4.2.4.2 Ethnopharmaceuticals

As previously mentioned, there are several plants that have and are being used traditionally as herbal remedies/medicines to treat ailments and diseases such as respiratory (coughs, colds, influenza, tuberculosis, asthma) and gastrointestinal (diarrhoea, stomach ache, dysentery) problems, cancer, urinary tract infections, etc. (Watt and Breyer-Brandwijk, 1962; Hutchings *et al*., 1996; van Wyk *et al*., 1997; van Wyk and Gericke, 2000; van Wyk and Wink; 2004, van Wyk, 2008). Due to the increased use of plants as herbal remedies/medicines, there is a need to determine and evaluate their potential toxic effects to the reproductive system.

Scientifically, several plants have been shown to have androgenic (Gautharman *et al*, 2002; Yakubu *et al*, 2008) and anti-androgenic (Hiremath *et al*, 1997; Gupta *et al*, 2006) properties by increasing or decreasing testosterone levels. Due to the androgenic and anti-androgenic properties of some plants, there is increased interest into research and development of plant-based fertility and contraceptive drugs.

Plants such as *Tribulus terrestis* and *Massularia acuminata*, which have been used as herbal aphrodisiac medicines, have androgen increasing properties (Gautharman *et al*, 2002; Yakubu *et al*, 2008). Leaf extracts of *Tricophus zeylanicus* administered to male mice increased

sexual behaviour (Subramoniam *et al*, 1997). *Montanoa tomentosa* (Carro-Juárez *et al*, 2004) and *Butea frondosa* (Ramachandran *et al*, 2004) have also been shown to increase sexual performance in male rats.

Striga orobanchioides (Hiremath *et al*, 1997) and *Albizia lebbeck* (Gupta *et al*, 2006) caused anti-androgenic effects in male rats by decreasing reproductive organ weights (testis, seminal vesicles, epididymis and prostate), sperm motility and density, and testosterone concentrations. *Hibiscus sabdariffa*, a plant claimed to be an aphrodisiac, decreased sperm count and caused hyperplasia of the testis in rats (Orisakwe *et al*, 2004).

1.4.2.5 Natural hormones

17β-Estradiol (E2), Estriol (E3) and Estrone (E1) are naturally occurring estrogenic hormones (Bjerselius *et al* 2001; Jiang *et al*, 2005), of which E2 is the main estrogen (Pawlak and Wiebe *et al*, 2007; Xuefei *et al*, 2007). These estrogenic hormones are the most potent of all EDCs (Pojana *et al*, 2007). These hormones regulate several biological processes during various stages of reproduction (Jonošek *et al*, 2006). E2 is also responsible for other processes within the body which include metabolism, cell proliferation, etc. (Tsai *et al*, 1994). As with many pharmaceuticals, natural estrogens are excreted in the urine of both humans and wildlife which then contaminate aquatic ecosystems (Julius *et al*, 2007). These hormones have been found in sewage effluents, river and drinking water (Xuefei *et al*, 2007) as well as in treated and untreated industrial and municipal wastewater (Pojana *et al*, 2007).

Exposure to estrogenic hormones can cause several adverse effects. Male *Xenopus laevis* (African-clawed frogs) exposed to E2 displayed demasculinization and testicular

development disruption (Hecker *et al*, 2005). Demasculinization was also observed in male Japanese quail exposed to E2, E1 and E3 (Whitsett *et al*, 1977). E2 treatment also inhibited testosterone production in hypophysectomised rats (Melner and Abner, 1980).

1.4.3 EDC monitoring/screening

The growing concern of adverse effects as result of EDC exposure in humans and wildlife as well as the increased incidence of EDC contamination has prompted extensive research into the development and validation of screening tests to detect and monitor EDCs in the environment and screen new substances for endocrine-disrupting capability. These screening tests involve assessing the effect of known and potential EDCs on reproductive function, sexual development and hormone production (EPA, 2005). Several animal models are being used as bio-indicators to monitor EDCs. These include frog species such as *Xenopus laevis* (African-clawed frog) (Cong *et al*, 2006), fish species such as *Oreochromis mossambicus* (Mozambique tilapia) (Riley *et al*, 2004), bird species such as *Falco peregrinus* (peregrine falcon) (Jiménez *et al*, 2007), as well as mammalian species such as *Mus musculus* (CD-1 mice) (Ji *et al*, 2010) and *Rattus norvegicus* (albino rats) (Kumar *et al*, 2008).

To assess the effect of EDCs on the reproductive system different methods are employed which include *in vitro*, *in vivo* and *ex vivo* methods. *In vitro* and *in vivo* methods are often used in conjunction with each other as a Tiered approach where Tier 1 is an *in vitro* and an *in vivo* screen and Tier 2, an *in vivo* screen. *In vivo* methods have been employed extensively to monitor the effects of EDCs. These methods have been suggested not to be suitable as a screening tool due to high costs, labour intensiveness, and the requirement for large numbers of animals as well as highly specialized equipment and skills. *In vitro* methods have thus

been suggested as an alternative screening tool for EDC monitoring due to low costs, reduced number of animals, standard equipment and the ability to screen a large number of samples with multiple endpoints (EPA, 2005).

There are several *in vitro* methods to screen for EDCs with regards to the male reproductive system which include the whole testis method, where perifusion, perfusion or whole organ incubation is employed; the sectioned or minced testes method; and the isolated and cultured cell method (EPA, 2005). Of these *in vitro* methods, the sectioned or minced testes method has been recommended by the EPA as a potential screening tool for EDC screening/monitoring due to low costs, use of standard and basic equipment and skills as well as being quick and simple to perform (EPA, 2005).

The aim of this study is to employ a minced testes method to screen environmental samples for male reproductive toxicity. The minced testes method which we will be employing in this study will involve the aseptic removal of the testes from male BALB/C mice. The testes will then be minced and the cells cultured. The cells will thereafter be incubated with a stimulant (LH) as well as the sample/s that we want to screen. Following this incubation period, hormone production (testosterone and estradiol) as well as cell viability will be assessed to determine the effect of the sample/s on the reproductive system.

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CHAPTER 2

The optimization of an *in vitro* testicular cell culture assay

2.1 Abstract

The aim of this study was to optimize an *in vitro* testicular cell culture assay by determining both optimal luteinizing hormone (LH) concentration and incubation time needed for testosterone production. Male Balb/c mice were sacrificed by cervical dislocation, testes removed and testicular cell cultures prepared. Cells were treated with varying concentrations of LH (10, 1, 0.1, 0.01 and 0 mu/ml) for 4 hours or 20 hours. Testosterone production was evaluated for each incubation period. Treatment of cells with LH (0.01 - 10 mu/ml) significantly increased ($P < 0.05$) testosterone production as compared to the control. Testosterone production did not differ significantly ($P > 0.05$) with respect to LH concentrations used in the treatments for both incubation periods. From the data obtained we have thus selected the 4 hour incubation period as well as the LH concentration of 10 mu/ml as optimal for the testicular cell culture assay.

2.2 Introduction

Endocrine disrupting chemicals (EDCs) are widespread within the environment (Ishibashi *et al*, 2001), many of which cause adverse effects on the male reproductive system of both humans and wildlife (Clotfelter *et al*, 2004) such as infertility, demasculinization, testicular cancer, decreased libido, abnormal reproductive function and sexual behaviour, etc. (Gray Jr., 1998; Ong *et al*, 2002; Snyder, 2003). It is thus important that screening tools be developed, validated and implemented to detect and monitor EDCs in the environment as well as to screen new substances with endocrine-disrupting capability.

The EPA recommends an *in vitro* minced testes (mammalian) method as a suitable screening tool for EDC screening/monitoring. This recommendation is based on the fact that this method, as compared to other *in vitro* and *in vivo* methods using a mammalian model, is cost-effective, minimizes animal usage, requires the use of standard and basic equipment, is quick and simple to perform, very sensitive and has the ability to screen many samples with multiple endpoints. The basic protocol for this method includes the following: (1) aseptic removal of testes; (2) testes minced and cell culture prepared; (3) cells incubated with stimulant together with sample/s to be screened; and (4) assess endpoints (hormone production, cell viability, etc.) (EPA, 2005).

In order to employ such a method, optimization of the protocol is required such as the determination of optimal stimulant concentration as well as the incubation time needed. The aim of this study is thus to optimize an *in vitro* testicular cell culture assay by determining both optimal stimulant (LH) concentration and incubation time needed for testosterone production.

2.3 Materials and methods

2.3.1 Reagents and chemicals

All chemicals, reagents, solvents were purchased from Sigma (Germany), unless otherwise stated in the text. All reagents were of analytical grade.

2.3.2 Animals

Three month old, pathogen-free, male Balb/C mice were used for this study. Mice were purchased from the University of Cape Town Animal Unit (Cape Town, South Africa). The mice were housed in a well-ventilated animal house with a 12 hour light/dark cycle and fed standard mouse feed (Medical Research Council, Cape Town, South Africa) with free access to normal drinking water.

2.3.3 Cell culture

Mice were sacrificed by cervical dislocation. The testes were subsequently removed (aseptically), minced and then suspended in 10 ml serum-free medium which consisted of 1 % glutamax (Invitrogen), 1 % Penicillin/Streptomycin/Fungizone mix (Sigma), 0.2 % bovine serum albumin (BSA), and RPMI-1640 medium (Sigma). After allowing debris to settle, the cells were transferred to a new tube together with subsequent serum-free medium resulting in a final volume of 10 ml. The cells were thereafter incubated at 37 ˚C with 5 % CO_2 for 1 hour. Following the incubation period, the cells were centrifuged at 1000 x g for 10 minutes.

The supernatant was then discarded and the cell pellet was resuspended in 10 ml serum-free medium and incubated at 37 ˚C with 5 % CO_2 for 30 minutes. The cells were centrifuged at 1000 x g for 10 minutes and the supernatant obtained was again discarded. The cell pellet was then resuspended in 10 ml serum-free medium to an approximate concentration of 3.4 x 10^6 cells/ml which was then used for cell culture assays.

2.3.4 Determination of optimal Luteinizing hormone (LH) concentration and incubation period

To determine the optimal LH concentration as well as incubation time needed for the cell culture assay, cells were seeded in 1.5 ml microtubes at a volume of 200 µl per tube. Varying concentrations of LH (10, 1, 0.1, 0.01 and 0 mu/ml) were added to the cell cultures at a volume of 200 µl per tube which were then incubated at 37 ˚C with 5 % CO_2. One set for 4 hours and the other, 20 hours. Following the 4 hour and 20 hour incubation periods, the cells were centrifuged at 1000 x g for 5 minutes and the supernatants obtained were assayed for testosterone concentrations using commercially available enzyme-linked immunosorbant (ELISA) kits (DRG Instruments, GmbH, Germany). The assays were performed as per manufacturer's instructions and the range of the testosterone was between 0 – 16 ng/ml.

2.3.5 Statistical analysis

Each experiment was performed two times in triplicate. Data was statistically analysed via one-way analysis of variance (ANOVA) followed by the Tukey's test using the SigmaStat 3.5 software package (Systat Software Inc., USA). (differences when $P < 0.05$, were considered as statistically significant).

2.4 Results

2.4.1 Testosterone production

The standard curve for the Testosterone ELISA is presented in Figure 2.1. A good inverse correlation (R^2 = 0.99) between percentage of maximum optical density and testosterone concentrations was obtained (Figure 2.1). LH-treatment of cells (0.01 - 10 mu/ml) for both 4 hour and 20 hour incubation periods significantly increased ($P < 0.05$) testosterone production as compared to the control (Figure 2.2). From the 4 hour to the 20 hour incubation period, an 11-fold and a 7-fold increase in testosterone production was observed for the control and the LH-treatments (0.01 – 10 mu/ml) respectively (Figure 2.2). No significant difference in testosterone production was observed for cells treated with LH (0.01 - 10 mu/ml) as compared to each other for both incubation periods (Figure 2.2).

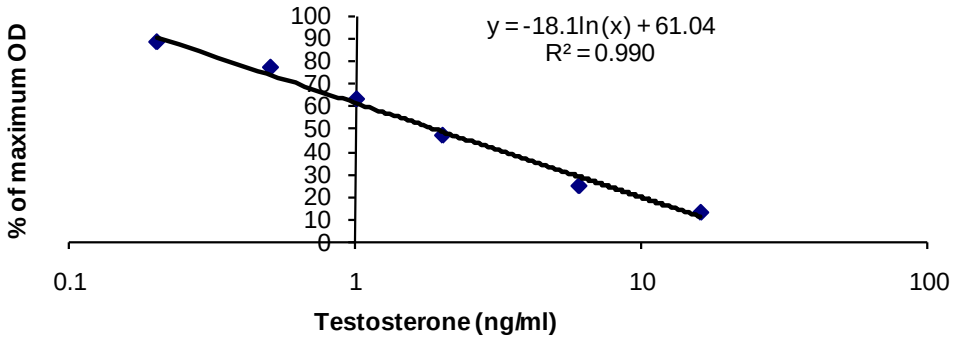

Figure 2.1 Standard curve of Testosterone ELISA.

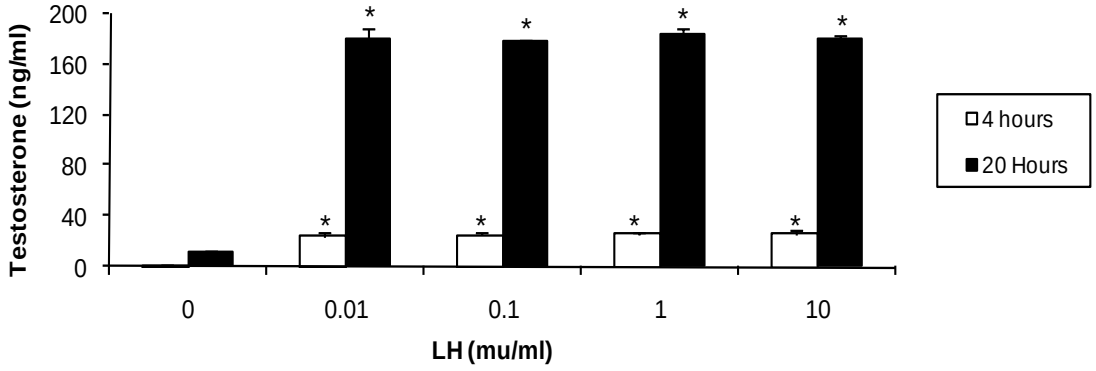

Figure 2.2 Effect of LH treatment on testosterone production at 4 hour and 20 hour incubation periods (n = 3). (* indicates $P < 0.05$ relative to the control)

2.5 Discussion

The rationale for using LH as a stimulant for testosterone production is based upon the biological process by which testosterone production occurs. LH from the anterior pituitary gland binds to LH-receptors on Leydig cells within the testes (Mader, 2001). The steroidogenesis pathway is then set off in which cholesterol is converted to testosterone by several enzymatic reactions (Seeley *et al*, 2003). Any change in this biological pathway can result in a testosterone imbalance which may cause adverse reproductive effects (EPA, 2005).

The present study set out to optimize an *in vitro* testicular cell culture assay by determining the optimal stimulant (LH) concentration and incubation time needed for testosterone production. LH treatment was shown to significantly increase testosterone production at all concentrations tested for both incubation periods as compared to the controls. However, there was no significant difference in testosterone production between the various LH concentrations tested. For both incubation periods there was a clear differentiation between

LH-treated and untreated cells. These findings show that both incubations periods as well as the various LH concentrations tested are effective for testosterone production. We have thus selected the 4 hour incubation period and LH concentration of 10 mu/ml as optimal for the *in vitro* testicular cell culture assay.

2.6 Conclusion

The results obtained from this study have shown that LH (0.01 – 10 mu/ml) significantly increased testosterone production for both the 4 hour and 20 hour incubation periods and that there is a clear differentiation between LH-treated and untreated cells. The 4 hour incubation period and LH concentration of 10 mu/ml have thus been selected as optimal for the *in vitro* testicular cell culture assay.

2.7 References

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CHAPTER 3

The effect of *Tulbaghia violacea* extracts on testosterone secretion by testicular cell cultures

3.1 Abstract

This study aimed to determine the effect of *Tulbaghia violacea* Harv. on the male reproductive system *in vitro* by using testicular cell cultures. *T. violacea* is a plant species indigenous to southern Africa and is used locally as a herbal remedy/medicine to treat several ailments. A 50 % ethanol extract of *T. violacea* was prepared. Three month old male Balb/C mice were sacrificed and testicular cell cultures were prepared. Cells were then treated with varying concentrations of the *T. violacea* ethanol extract (with/without Luteinizing hormone (LH)-treatment) and incubated for 4 hours. Hormone production and cell viability were evaluated. Treatment of cells with *T. violacea* (312.5 - 5000 µg/ml) significantly increased ($P < 0.05$) LH-induced testosterone production as compared to vehicle-treated control (DMSO) whereas cells without LH-treatment showed no significant change in testosterone concentrations. No significant effect on cell viability was observed at all concentrations tested. The data presented shows that *T. violacea* has androgenic properties. Further studies are warranted to determine and clarify the exact mechanisms involved.

3.2 Introduction

Tulbaghia violacea Harv. is a plant species belonging to the Alliaceae family, indigenous to South Africa and has been used as a herbal remedy/medicine to treat several ailments which include respiratory diseases (tuberculosis and asthma), oesophageal cancer, gastrointestinal problems as well as colds and fever (van Wyk *et al*, 1997). It is popularly known as wild garlic, society garlic or sweet garlic and the leaves have also been used to treat sinus headaches and as a deterrent for moles in gardens due to the strong garlic smell (Kubec *et al*, 2002). The Zulus, who refer to *T. violacea* as isihaqa, have used this plant as an aphrodisiac medicine (Dyson, 1998) as well as a snake repellant (Kubec *et al*, 2002). Studies have shown, however, that excessive consumption of *T. violacea* can have adverse effects which include gastroenteritis, abdominal pain and inflammation (van Wyk *et al*, 1997; van Wyk and Gericke, 2000).

Since *T. violacea* has been reported to being used as an aphrodisiac medicine and as remedy for several ailments, scientific evidence is needed to determine and evaluate the potential androgenic activity of this plant. To our knowledge, no studies have been conducted to assess the effect of *T. violacea* on the reproductive system. The aim of this pilot study is thus to determine the effect of *T. violacea* on the male reproductive system *in vitro* by assessing testosterone and estradiol production, as well as cell viability.

3.3 Materials and methods

3.3.1 Reagents and chemicals

All chemicals, reagents, solvents were purchased from Sigma (USA), Merck (Germany) and Roche Diagnostics (South Africa) and all reagents were of analytical grade.

3.3.2 Animals

After obtaining approval from the institutional animal ethical committee, male Balb/C mice were used for this study. Mice were purchased from the University of Cape Town Animal Unit (Cape Town, South Africa) and were pathogen free. The mice were then housed in a well-ventilated animal house with a light/dark cycle of 12:12. The mice had free access to normal drinking water and were fed standard mouse feed (Medical Research Council, Cape Town, South Africa).

3.3.3 Cell culture

Three month old mice were sacrificed by cervical dislocation. The testes were then removed aseptically, minced and then transferred to a tube (Greiner Bio-one) containing 10 ml serum-free medium (0.2 % bovine serum albumin (BSA), 1 % glutamax (Invitrogen), 1 % Penicillin/Streptomycin/Fungizone mix (Sigma) and RPMI-1640 medium (Sigma)). Debris was allowed to collect at the bottom of the tube and thereafter, the supernatant (containing cells) was transferred to a new tube. Subsequent serum-free medium was added to the cells

resulting in a final volume of 10 ml. The cells were then incubated at 37 ˚C with 5 % CO_2 for 1 hour. After incubation, the cells were centrifuged at 1000 x g for 10 minutes. The supernatant was then discarded and the cells were resuspended in 10 ml serum-free medium and incubated at 37 ˚C with 5 % CO_2 for 30 minutes. The cells were centrifuged as before and the supernatant obtained was again discarded. The cell pellet was then resuspended in 10 ml serum-free medium to an approximate concentration of 3.4 x 10^6 cells/ml and then used for cell viability and hormone production determinations.

3.3.4 Preparation of *T. violacea* ethanol extract

The voucher specimen of *Tulbaghia violacea* Harv. was identified by the University of the Western Cape Herbarium, Bellville, South Africa. Fresh plant organs (50 g) of *T. violacea* (leaves and rhizomes) were homogenized in a Waring blender and extracted overnight in a sealed container at room temperature in ethanol (100 ml) to obtain a 50 % (500 mg/ml) extract. The 50 % ethanol extract was subsequently filtered through Whatman No. 4 qualitative filter paper to remove any remaining plant material and thereafter air-dried at room temperature. The extract was then reconstituted to 50 % (w/v) in DMSO and stored at 4 ˚C until use. Dilutions of the extract were made in DMSO and these were used for subsequent assays.

3.3.5 *T. violacea* and Luteinizing hormone (LH) treatment of cells

Cells were seeded in a 96-well tissue culture plates (Nunc, Serving Life Science, Denmark) at a volume of 50 µl per well. Varying concentrations of *T. violacea* (5000, 2500, 1250, 625, 312.5, 156.25 and 0 µg/ml) were added to the cell cultures at a volume of 1 µl per well and

the culture plates were then incubated at 37 ˚C with 5 % CO_2 for 1 hour. Cells that received Dimethyl sulphoxide (DMSO) in the medium were used as a vehicle-treated control, throughout the study. The cells were thereafter incubated in the presence and absence of LH (10 mu/ml) (50 µl per well) at 37 ˚C with 5 % CO_2 for 4 hours.

3.3.6 Hormone production

After the 4 hour incubation period, supernatant from LH-treated and non-treated cells were assayed for testosterone and estradiol concentrations using commercially available ELISA kits (DRG Instruments, GmbH, Germany) to assess the effect of *T. violacea* on hormone production. The assays were performed as per manufacturer's instructions. The range of the testosterone and estradiol assays were between 0 – 16 ng/ml and 9.7 – 2000 pg/ml respectively.

3.3.7 Cell viability

The effects of *T. violacea* on cell viability were determined by XTT assay. In this assay, the reduction of the yellow tetrazolium salt (XTT) to an orange formazan product by viable cells, were measured. Cells were seeded in a 96-well tissue culture plate (Nunc, Apogent, Denmark) with varying concentrations of *T. violacea* in the presence and absence of LH, as previously described. At 1 hour incubation, XTT reagent mix (Roche Diagnostics GmbH, Germany) was added to the culture plate at a volume of 50 µl per well and incubated at 37 ˚C with 5 % CO_2 for 4 hours. Formazan formation was then spectrophotometrically quantified at 492 nm with a microtitre plate reader (Multiskan Ex, Thermo Electron Corporation).

3.3.8 Statistical analysis

SigmaStat software (Systat Software Inc., USA) was used for statistical analysis. Each experiment was performed thrice in quadruplicate and data was statistically analysed via one-way ANOVA ($P < 0.001$) and regression analysis.

3.4 Results

3.4.1 Effects of *T. violacea* on cell viability

Treatment of cells with varying concentrations of *T. violacea* (with/without LH-treatment) had no significant effect on cell viability as compared to control (Figure 3.1).

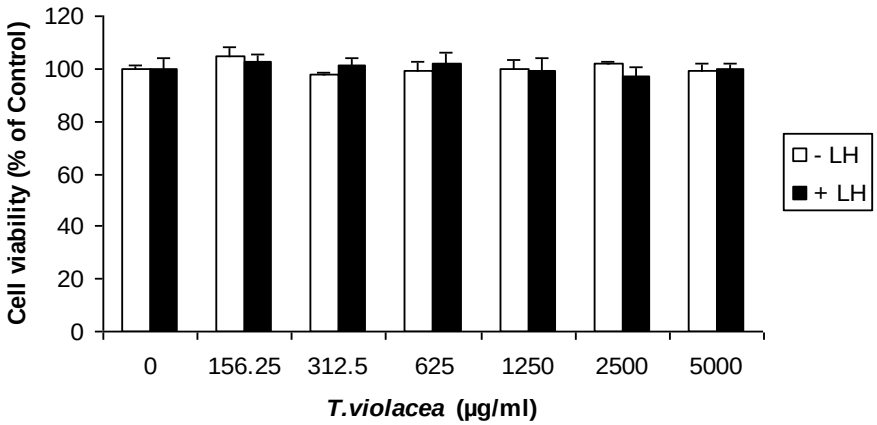

Figure 3.1 Cell viability of testes cell suspension treated with varying concentrations of *T. violacea* (with/without LH-treatment).

3.4.2 Hormone production

T. violacea significantly increased ($P < 0.05$) LH-induced testosterone secretion at concentrations 312.5 - 5000 µg/ml as compared to control (Figure 3.2). Cells incubated in the absence of LH secreted low levels of testosterone and showed no significant effect as compared to control (Figure 3.2). In LH-stimulated cultures, treatment with *T. violacea* resulted in a 30 - 72 % increase in testosterone production as compared to control. Estradiol production was undetectable at all concentrations tested (with/without LH-treatment) (data not shown).

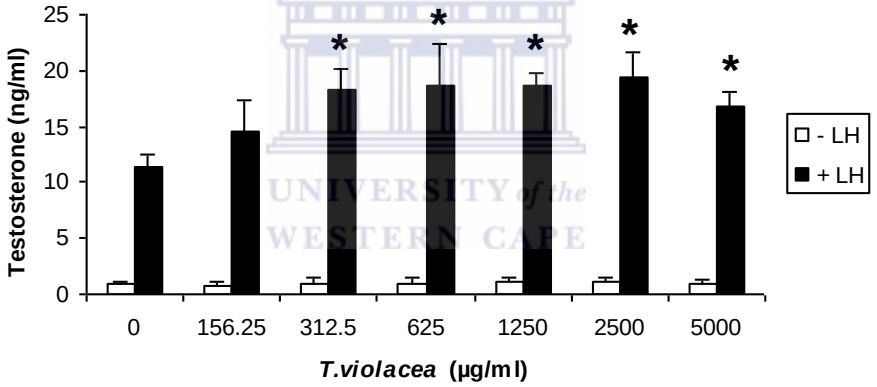

Figure 3.2 Effect of *T. violacea* on testosterone secretion (with/without LH-treatment). Cells were treated for 4 hours and supernatants were screened thereafter for testosterone. (* indicates $P < 0.05$ relative to the control)

3.5 Discussion

The present study investigated the effect of *T. violacea* on the male reproductive system *in vitro* at the testicular level. To assess the potential androgenic or anti-androgenic effect of the

plant extract on the reproductive system, cell viability and hormone production were investigated.

Normal functioning of the reproductive system is essential for normal sexual development, behaviour, spermatogenesis, etc. Under normal conditions, testosterone, the main sex hormone in males, is produced in the testes by the Leydig cells through steroidogenesis upon stimulation by LH from the pituitary gland (Gail and Hedger, 1992; Kumar *et al*, 2008). Any variation in this biochemical pathway can result in an inhibition or stimulation of sex hormone synthesis, resulting in a hormonal imbalance which can cause adverse effects on the reproductive system (EPA, 2005).

A deficiency in testosterone production can have major adverse effects on reproductive health as well as other bodily functions and processes. Testosterone deficiency has been associated with aging (Morley and Perry III, 2000; El-Sakka and Hassoba, 2006) as well as with several diseases and disorders which include metabolic syndrome, hypogonadism, osteoporosis, obesity, diabetes mellitus type II, erectile dysfunction, Alzheimer's disease and cardiovascular disease (Jones, 2007; Schulman *et al*, 2009). Effects of low testosterone levels include decreased libido and low sperm count (Zitzmann, 2008), infertility, decreased muscle mass and strength, cognitive impairment and depression (Morley and Perry III, 2000; Zitzmann and Nieschlag, 2000; Schulman *et al*, 2009). Normalization of testosterone levels is thus imperative to maintain and improve reproductive health and quality of life.

Testosterone replacement therapy has been opted as a treatment for several of the previously mentioned diseases, disorders and effects (Morley and Perry III, 2003; Raynaud, 2009) and is available in oral, intramuscular, buccal, subdermal, and transdermal preparations (Schulman

et al, 2009). There are several issues, however, concerning the above mentioned available treatments such as high cost, difficulty of administration and side-effects (Bouloux, 2005; Srinivas-Shankar and Wu, 2005).

The present study shows that the ethanol extract of *T. violacea* increased testosterone synthesis which indicates its androgenic activity. The increase in testosterone concentrations could possibly be due to an increase in steroidogenesis (increased enzymatic conversion of cholesterol to testosterone). Furthermore, the extract could be enhancing the action of LH or possibly increasing the response of Leydig cells to LH by increasing membrane-bound LH receptor expression. Unstimulated cells showed no significant effect in testosterone concentrations indicating that the extract does not stimulate testosterone production independently of LH. These findings suggest that *T. violacea* may potentially be used as a supplement to stimulate testosterone production, thereby maintaining and improving reproductive health and quality of life.

3.6 Conclusion

The results obtained from this pilot *in vitro* study have demonstrated that the ethanol extract of *Tulbaghia violacea* has androgenic properties by significantly increasing LH-induced testosterone production in mouse testes with no significant change in cell viability. Further studies are warranted to determine and clarify the exact mechanisms involved.

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CHAPTER 4

The effect of *Sutherlandia frutescens* and *Artemisia afra* extracts on the male reproductive system *in vitro*

4.1 Abstract

This study aimed to determine the effect of *Sutherlandia frutescens* and *Artemisia afra* on the male reproductive system *in vitro* by using testicular cell cultures of Balb/c mice. Ethanol extracts of each plant was prepared and cells were treated with varying concentrations of each extract (0, 156.25, 312.5, 625, 1250, 2500 and 5000 µg/ml) with or without Luteinizing hormone (LH) treatment and incubated for 4 hours. Cytotoxicity by lactate dehydrogenase (LDH) measurement and hormone production (testosterone and estradiol) were endpoints that were evaluated. Cells treated with *S. frutescens* (156.25 - 5000 µg/ml) and *A. afra* 156.25 - 2500 µg/ml) showed no significant effect in LH-induced testosterone production. Treatment of cells with *A. afra* significantly decreased LH-induced testosterone production (43 %) at 5000 µg/ml. Cells incubated in the absence of LH secreted low levels of testosterone at all concentrations tested. No significant effect in estradiol production or cytotoxicity was observed with or without LH-treatment at all concentrations tested. The data presented shows that *S. frutescens* and *A. afra* are not toxic to testicular cells *in vitro* and that *A. afra* decreases testosterone production at high concentrations. Further studies into the exact mechanisms involved are warranted.

4.2 Introduction

Southern Africa is home to a rich plant biodiversity representing approximately 25 % of all higher plants in the world (van Wyk, 2008). Many of these plants have been and are being used traditionally to treat several diseases and ailments such as respiratory (coughs, colds, influenza, tuberculosis, asthma) and gastrointestinal (diarrhoea, stomach ache, dysentery) problems, cancer, urinary tract infections, etc. (Watt and Breyer-Brandwijk, 1962; Hutchings *et al*., 1996; van Wyk *et al*., 1997; van Wyk and Gericke, 2000; van Wyk and Wink, 2004; van Wyk, 2008). *S. frutescens* and *A. afra* are two such plant species that are indigenous to South Africa and are being used traditionally as herbal remedies/medicines. Both *S. frutescens* and *A. afra* have a long history of medicinal use amongst several cultural groups which include the Xhosa, Zulu, Cape Dutch and Khoi-san (van Wyk and Albrecht, 2008).

S. frutescens (L.) R.Br., commonly known as cancer bush, has been used traditionally to treat internal cancers, fever, eye infections, diabetes, stress, gastrointestinal problems, pains and wounds (Stander *et al*, 2007; van Wyk and Albrecht, 2008) as well for the improvement of overall health of HIV/AIDS patients (Tai *et al*, 2004; Stander *et al*, 2007). *S. frutescens* belongs to the Fabaceae family and is distributed along the west coast of the Western Cape (van Wyk, 1997; Chinkwo, 2005). This plant is also indigenous to Lesotho, southern Namibia and southeastern Botswana (van Wyk and Albrecht, 2008). *S. frutescens* is also known as kankerbos (Afrikaans), motlepelo (Sotho) insiswa (Zulu), and unwele (Xhosa) (van Wyk and Albrecht, 2008). Scientifically, *S. frutescens* has been shown to have potential antioxidant (Fernandes *et al*, 2004), anti-HIV (Harnett *et al*, 2005), stress relieving (Prevoo *et al*, 2008), and anti-cancer (Tai *et al*, 2004) properties.

A. afra Jacq. ex Willd., commonly known as African wormwood and wilde-als, belongs to the Asteraceae family with a wide distribution ranging from the Cape to as far as East Africa and Ethiopia (Mukinda and Syce, 2007; Van de Kooy *et al*, 2008; van Wyk, 2008). It is used traditionally to treat gastrointestinal problems, colds, fever, influenza and malaria and has also been used as a painkiller, an anthelmintic and as eye drops to treat eye infections (van Wyk, 2008; Van de Kooy *et al*, 2008). The Zulu and Xhosa people, who refer to *A. afra* as Umhlonyane, have also used this plant to treat Dysmenorrhoea (van Wyk and Gericke, 2000), a menstrual disorder characterized by severe uterine pain (Deb and Raine-Fenning, 2008). Scientifically, *A. afra* has been shown to have antibacterial (Mangena and Muyima, 1999; Mativandlela *et al*., 2008), antifungal (Huffman *et al*, 2002; Mangena and Muyima, 1999), antioxidant (Naidoo *et al*, 2008) and antidepressant (Nielson *et al*, 2004) activities as well as spasmolytic properties (Mulatu and Mekonnen, 2007).

To our knowledge, no scientific data is yet available on the effect of *S. frutescens* and *A. afra* on the reproductive system. Since these plants are being used medicinally, there is a need to determine and evaluate their potential negative effects on the body. The aim of this pilot study is thus to determine the effect of *S. frutescens* and *A. Afra* on the male reproductive system *in vitro* by looking at several endpoints which include cytotoxicity and steroid hormone production (testosterone and estradiol).

4.3 Materials and methods

4.3.1 Chemicals

All chemicals were purchased from Sigma (Germany) and were of analytical grade.

4.3.2 Animals

Three month old, pathogen free, male Balb/C mice were purchased from the University of Cape Town Animal Unit (Cape Town, South Africa) and were housed in a well-ventilated animal house with a 12:12 light/dark cycle. The mice had free access to tap water and were fed standard mouse feed (Medical Research Council, Cape Town, South Africa). One mouse was used per experiment.

4.3.3 Cell culture

Mice were sacrificed by cervical dislocation. The testes were then removed aseptically, minced using scissors and suspended in 10 ml serum-free medium (0.2 % bovine serum albumin (BSA), 1 % Penicillin/Streptomycin/Fungizone mix (Sigma), 1 % glutamax (Invitrogen), and RPMI-1640 medium (Sigma)) in a tube (Greiner Bio-one). Settled debris was discarded and the supernatant (containing testicular cells) was transferred to a new tube together with subsequent serum-free medium resulting in a final volume of 10 ml. The cells were thereafter incubated in an incubator at 37 ˚C and 5 % CO_2 for 1 hour. Following the incubation period, the cells were centrifuged at 1000 x g for 10 minutes. After discarding the

supernatant, the cell pellet was resuspended in 10 ml serum-free medium and incubated at 37 ˚C and 5 % CO_2 for a further 30 minutes. The cells were subsequently centrifuged at 1000 x g for 10 minutes and the supernatant was discarded. The cell pellet was then resuspended again in 10 ml serum-free medium to an approximate concentration of 3.4 x 10^6 cells/ml which was then used for cell cultures.

4.3.4 Preparation of *S. frutescens* and *A. afra* ethanol extracts

The voucher specimens of *S. frutescens* and *A. afra* were identified by the University of the Western Cape Herbarium, Bellville, South Africa. Leaves and rhizomes (total weight of 50 g) of each plant were homogenized in a Waring blender and extracted overnight at room temperature in a sealed container with 100 ml ethanol to obtain a 50 % (500 mg/ml) extract. The extract was subsequently filtered through Whatman No. 4 qualitative filter paper and thereafter air-dried at room temperature. The extract was then reconstituted to 50 % (w/v) in dimethyl sulphoxide (DMSO) and stored at 4 ˚C until use. Dilutions of the extract were made in DMSO and these were used for subsequent assays.

4.3.5 Plant extracts and Luteinizing hormone (LH) treatment of cells

Cells were seeded in a 96-well tissue culture plate (Nunc, Serving Life Science, Denmark) at a volume of 50 µl per well. Varying concentrations of *S. frutescens* and *A. afra* (5000, 2500, 1250, 625, 312.5, 156.25 and 0 µg/ml) diluted in DMSO were added to the cell cultures at a volume of 1 µl per well and the culture plate was then incubated at 37 ˚C in 5 % CO_2 for 1 hour. The cells were thereafter incubated in the presence and absence of 10 mu LH per ml in serum free medium (50 µl per well) at 37 ˚C in 5 % CO_2 for 4 hours.

4.3.6 Cytotoxicity determination

Cytotoxicity of *S. frutescens* and *A. afra* was assessed by lactate dehydrogenase (LDH) measurement. Following the 4 hour incubation period, supernatants from LH-treated and non-treated cells were transferred to a 96-well plate (Nunc, Serving Life Science, Denmark) at a volume of 10 µl per well. Cytotoxicity kit reaction mix (Cytotoxicity Detection kit, Biovision, USA) was then added at a volume of 100 µl per well and the plate was then incubated at room temperature for 1 hour. LDH was then spectrophotometrically quantified at 492 nm (before and after 1 hour incubation) with a microtitre plate reader (Multiskan Ex, Thermo Electron Corporation).

4.3.7 Hormone production

To assess the effect of *S. frutescens* and *A. afra* on testosterone and estradiol production, supernatant from LH-treated and non-treated cells (following the 4 hour incubation period) were assayed for testosterone and estradiol concentrations using commercially available enzyme-linked immunosorbant (ELISA) kits (DRG Instruments, GmbH, Germany). The assays were performed as per manufacturer's instructions. The detection range of the testosterone and estradiol assays were between 0 – 16 ng/ml and 9.7 – 2000 pg/ml respectively.

4.3.8 Statistical analysis

Each experiment was performed three times in triplicate and data was statistically analysed via one-way analysis of variance (ANOVA) followed by the Tukey's test using SigmaStat 3.5

software package (Systat Software Inc., USA). (differences when $P < 0.05$, were considered as statistically significant).

4.4 Results

4.4.1 Cytotoxicity

S. frutescens and *A. afra* treatment of cells (with/without LH-treatment) did not result in any significant toxic effect at all concentrations tested (data not shown).

4.4.2 Hormone production

A similar trend was seen in both *S. frutescens* and *A. afra*–treated cells (Figure 4.1). LH-induced testosterone production was slightly, but not significantly decreased at concentrations tested between 156.25 -5000 µg/ml (*S. frutescens*) (Figure 4.1A) and 156.25 - 2500 µg/ml (*A. afra*) (Figure 4.1B) as compared to control. However, *A. afra* significantly decreased LH-induced testosterone production (43 %) at 5000 µg/ml (Figure 4.1B). Cells incubated in the absence of LH secreted low levels of testosterone and no significant effect on testosterone production was observed. Estradiol production (with/without LH-treatment) was low at all concentrations tested (data not shown).

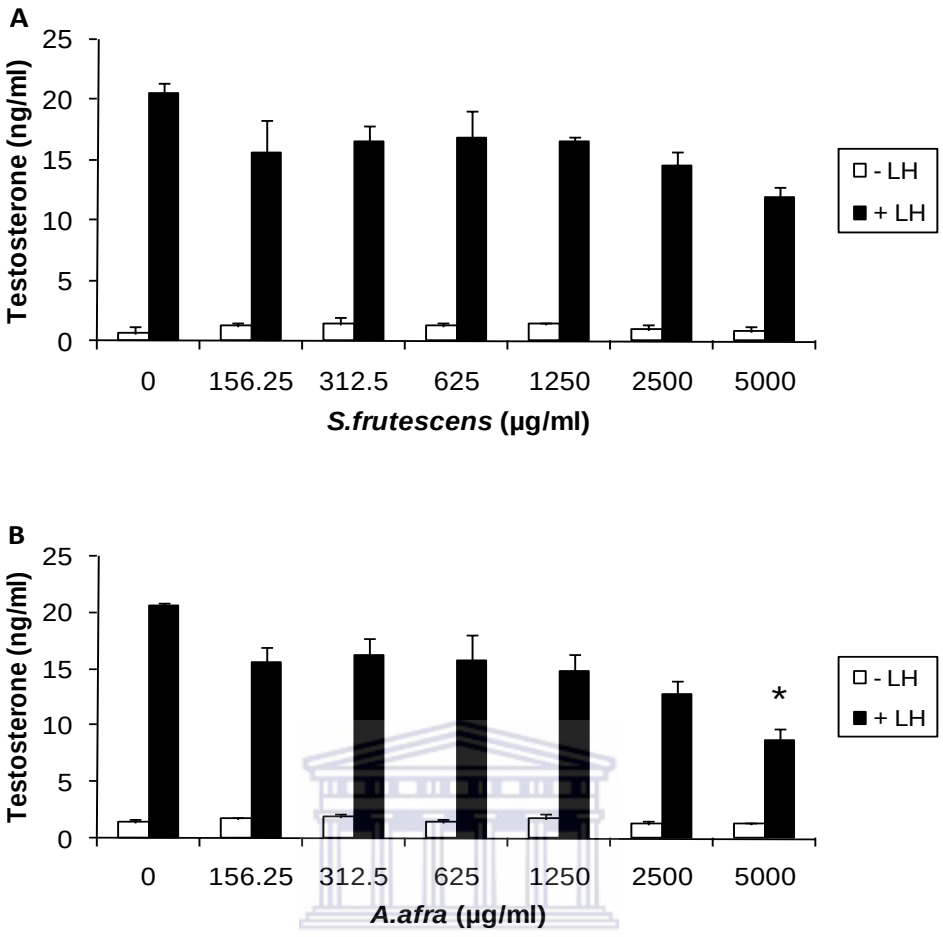

Figure 4.1 Effect of *S. frutescens* (A) and *A. afra* (B) on testosterone secretion (with/without LH-treatment). Cells were treated for 4 hours and supernatants were screened thereafter for testosterone. (* indicates $P < 0.05$ relative to the control)

4.5 Discussion

The age old and increasing use of plants as herbal remedies/medicines has prompted investigations into their effects on biological and physiological systems scientifically. As it stands, plant usage is based on anecdotal evidence and scientific data is needed to provide evidence and understanding of their effects as well as potential toxicity. The present study investigated the acute effect of *S. frutescens* and *A. afra* ethanol extracts on the male reproductive system *in vitro* by using testicular cell cultures of Balb/c mice. Cytotoxicity by

LDH measurement and hormone production (testosterone and estradiol) were endpoints that were assessed.

LDH measurement for cytotoxicity determination is based on the release of cellular LDH upon cell lysis due to toxicant exposure. *S. frutescens* and *A. afra* ethanol extracts did not result in any significant effect in LDH activity and are therefore not cytotoxic to testicular cells.

Testosterone is predominantly a male sex hormone produced in the testes by Leydig cells and is responsible for normal reproductive function, secondary sexual characteristics, development, spermatogenesis maintenance, sexual behaviour, etc. (Mader, 2001; Seeley *et al*, 2003; Yakubu *et al*, 2008). Testosterone production is as a result of steroidogenesis in Leydig cells upon stimulation by LH. LH is a hormone produced by the pituitary gland (Mader, 2001; Seeley *et al*, 2003). Upon stimulation by LH, the steroidogenic pathway begins with the formation of cyclic adenosine 3',5'-cyclic monophosphate (cAMP) and protein kinase A stimulation which in turn results in increased cholesterol utilization and transport by steroid acute regulatory protein (StAR) to the inner mitochondrial membrane where cholesterol is converted to testosterone by several enzymatic conversions. A testosterone imbalance can occur (increase or decrease) as a result of alterations in the above mentioned pathways which may then result in adverse effects to the reproductive system (EPA, 2005).

Testosterone measurement is widely used as an androgenic marker (Walton *et al*, 1995; Kumar *et al*, 2008; Yakubu *et al*, 2008) and has for this reason been employed in this study. Scientifically, several plants have been shown to have androgenic (Gautharman *et al*, 2002;

Yakubu *et al*, 2008) and anti-androgenic (Hiremath *et al*, 1997; Gupta *et al*, 2006) properties by increasing or decreasing testosterone levels. In this study, *S. frutescens* did not demonstrate any significant effect on testosterone production whilst *A. afra* displayed a significant decrease at the highest concentration tested thus indicating its anti-androgenic potential. The decrease in testosterone production may be due to an inhibition in the steroidogenesis pathway either by inhibiting cAMP, protein kinase A, StAR synthesis and cholesterol transport or steroidogenic enzymatic activity. These findings show that acutely, *S. frutescens* and *A. afra* are non-toxic to testicular cells and that *A. afra* decreases testosterone production at high concentrations.

4.6 Conclusion

This study demonstrates for the first time the effect of *S. frutescens* and *A. afra* on the male reproductive system *in vitro*. The results obtained show that the ethanol extracts of both plants are not cytotoxic to testicular cells and that *A. afra* decreases testosterone production at high concentrations. Further studies are warranted to elucidate the exact mechanisms regulating the decrease in testosterone production by *A. afra*.

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CHAPTER 5

The *in vitro* toxicity of Manganese, Copper, Cadmium, and Magnesium on mouse testes

5.1 Abstract

The effect of manganese, copper, cadmium and magnesium on the male reproductive system was assessed *in vitro* by using testicular cell cultures. Cells were treated with varying concentrations of each metal salt (200, 100, 50, 25, 12.5, and 6.25 µM) with or without Luteinizing hormone (LH) treatment and incubated for 4 hours. Endpoints evaluated included cell viability, testosterone and estradiol production. Cadmium and manganese treatment (with/without LH-treatment) resulted in a significant decrease in cell viability (21 – 59 % and 34 – 84 % respectively) at all concentrations tested. Copper significantly decreased cell viability at concentrations 25 – 200 µM (17 – 47 %) (without LH-treatment) and at concentrations of 6.25, 100 and 200 µM (23 – 48 %) (with LH-treatment). Magnesium (with/without LH-treatment) significantly increased cell viability (19 %) at a concentration of 25 µM. Cadmium significantly decreased LH-induced testosterone production (49 – 95 %) at all concentrations tested. Copper and magnesium significantly decreased LH-induced testosterone production at concentrations 200 µM (33 %) and 25 µM (26 %) respectively. No significant change in testosterone production was observed in cells treated with manganese. The data presented shows that manganese, cadmium and copper are highly toxic to testicular cells *in vitro* and therefore may potentially cause reproductive toxicity.

5.2 Introduction

Endocrine-disrupting compounds (EDCs) are exogenous compounds/chemicals which interfere with, or have adverse effects on the production, distribution and function of natural hormones, thereby affecting normal endocrine activity, health and quality of life of both humans and wildlife (Clotfelter *et al*, 2004). The reproductive system is highly susceptible to these compounds/chemicals due to it being controlled by an array of hormonal signals (Mills and Chichester, 2005). Several heavy metals have been found to have endocrine-disrupting capability and therefore may potentially have adverse effects on the reproductive system (Meeker *et al*, 2008). Heavy metal contamination result from various sources which include natural (mineral deposit weathering, brush burning, windblown dusts) and anthropogenic (mining, industry, smelting, hazardous waste sites, agriculture, etc.) activities (Calderón *et al*, 2003; Khan *et al*, 2008). A number of heavy metals are trace elements that are essential for physiological and biological processes but become toxic at high levels (Goldhaber, 2003; Dean *et al*, 2007; Meeker *et al*, 2008). Bioaccumulation of these heavy metals is particularly of great concern (Chary *et al*, 2008). Since a number of heavy metals have been shown to have endocrine-disrupting capability, it is thus important to determine and assess their effects on the reproductive system. The aim of this study is to determine the effect of four heavy metals, namely cadmium, manganese, copper and magnesium on the male reproductive system *in vitro* using cell viability and hormone production as biomarkers.

Cadmium (Cd) is a heavy metal which has been shown to cause adverse effects in several organ systems which include the liver, kidneys and testes (Åkesson, 2005; Kim and Soh, 2009; Siu *et al*, 2009), causing toxicity in both humans and animals (Kirkham, 2006; Thompson and Bannigan, 2008). It has also been shown to be carcinogenic (IARC, 1993;

Waalkes, 2000; Yamada *et al*, 2009) by inducing tumours in the prostate, testes and lungs (Waalkes, 2000; Zhou *et al*, 2004). Widespread in the environment, industrial Cd exposure is as a result of mining, smelting, combustion of fossil fuels (Nordberg *et al*, 1992; Zhou *et al*, 2004; Thompson and Bannigan, 2008), agricultural run-off, leechate from landfill sites (Thompson and Bannigan, 2008), battery and dye manufacturing and refining of metals (Siu *et al*, 2009). General population exposure to Cd is associated with contaminated drinking water, food and cigarette smoke (WHO, 2000; Zhou *et al*, 2004; ATSDR, 2008; Siu *et al*, 2009).

Manganese (Mn) is an essential trace element/nutrient for physiological processes (Elbetieha *et al*, 2001; Erikson *et al*, 2005; Rovetta *et al*, 2007) and an extensively used heavy metal in industry (Cheng *et al*, 2005) which is toxic at high concentrations/doses (Ponnapakkam, 2003; Vez´er *et al*, 2005). Exposure to Mn is as a result of both industrial, which include mining, welding, manufacturing of batteries and dyes, and alloy production, and environmental exposure which is usually due to contaminated drinking water and exposure to Mn-containing agricultural products (pesticides, fungicides and fertilizers) (Schneider *et al*, 2006). Chronic exposure to highly Mn-concentrated aerosols and dusts as well as the consumption of water contaminated with high levels of Mn can result in manganism, a Mn-induced neurotoxic disorder with symptoms similar to Parkinson's disease such as physcological disturbances and loss of motor function. Further symptoms include fatigue, lethargy, loss of appetite and sex drive, muscle cramps, and insomnia (Soldin and Aschner, 2007). Other toxic effects due to Mn exposure include liver and testicular damage as well as immune and reproductive system dysfunction (Elbetieha *et al*, 2001).

Copper (Cu) is an essential trace element important in several biological processes which include cellular respiration, iron metabolism and transport as well as oxidative stress protection (Kwok *et al*, 2008; Monteiro *et al*, 2009; Wang *et al*, 2009). Eventhough Cu is an essential element, it has toxic effects at high doses/concentrations (Kwok *et al*, 2008; Wang *et al*, 2009). Cu contamination of the environment is as a result of several anthropogenic activities which include Cu mining, industrial and agricultural processes (Krång and Ekerholm, 2006; Contreras *et al*, 2007; Kwok *et al*, 2008). Cu contamination of aquatic ecosystems is also as a result of industrial and municipal wastewaters and urban stormwaters (Cooper *et al*, 2009), as well as from Cu-based anti-fouling coating on ship hulls (Kwok *et al*, 2008). Cu is also used as a preservative in fish feeds (Dean *et al*, 2007). Adverse effects due to high levels of Cu exposure are associated with neurodegenerative and autosomal recessive disease (Wang *et al*, 2009), renal dysfunction (Galhardi *et al*, 2004), hepatic damage and gastrointestinal problems (Bradberry, 2007). Cu has also been shown to cause adverse effects in marine organisms such as toxicity in marine copepod *Tigropus japonicus* (Kwok *et al*, 2008), reduced mating behaviour in male shore crabs (*Carcinus maenas*) (Krång and Ekerholm, 2006) as well as toxicity in several fish species (Clearwater *et al*, 2002).

Magnesium (Mg) is an essential trace element which is vital to a range of physiological and biochemical processes and functions (Vormann, 2003; Gowda *et al*, 2004) such as adenosine triphosphate (ATP), calcium, potassium and carbohydrate metabolism (Frakes and Richardson, 1997). Mg is also used in industry (alloy manufacturing) (Mordike and Ebert, 2001) as well as in medicine where its use is suggested to be beneficial for the treatment of cardiological ailments, eclampsia, preclampsia and asthma (Frakes and Richardson, 1997). Mg has also been hypothesized as being beneficial for treatment-resistant depression (Eby III

and Eby, 2009) and shown to have a nephroprotective effect in patients undergoing cisplatin-based chemotherapy for epithelial ovarian cancer (Bodnar *et al*, 2008).

5.3 Materials and methods

5.3.1 Reagents and chemicals

All chemicals were purchased from Sigma (USA) and Merck (Germany) and were of analytical grade.

5.3.2 Animals

Three month old, pathogen free, male Balb/C mice were used for this study. The mice were purchased from the University of Cape Town Animal Unit (Cape Town, South Africa) and were housed in a well-ventilated animal house with a light/dark cycle of 12:12. The mice had free access to normal drinking water and were fed standard mouse feed (Medical Research Council, Cape Town, South Africa). One mouse was used for each experiment.

5.3.3 Cell culture

Mice were sacrificed by cervical dislocation. The testes were then removed aseptically and minced using scissors. The minced testes were then transferred to a tube (Greiner Bio-one) containing 10 ml serum-free medium (1 % glutamax (Invitrogen), 0.2 % bovine serum albumin (BSA), 1 % Penicillin/Streptomycin/Fungizone mix (Sigma) and RPMI-1640

medium (Sigma)). The cells were transferred to a new tube with subsequent serum-free medium to a final volume of 10 ml. The cells were then incubated at 37 ˚C and 5 % CO_2 for 1 hour. After incubation, the cells were centrifuged at 1000 x g for 10 minutes. The supernatant was discarded and the cell pellet was resuspended in fresh serum-free medium (10 ml) and incubated at 37 ˚C and 5 % CO_2 for an additional 30 minutes. The cells were subsequently centrifuged as previously described and the supernatant obtained was discarded. The cell pellet was then resuspended in serum-free medium (10 ml) to a concentration of 3.4 x 10^6 cells/ml and then used for cell cultures.

5.3.4 Preparation of heavy metal test solutions

Inorganic salts ($CuSO_4.5H_2O$, $3CdSO_4.8H_2O$, $MnSO_4.H_2O$ and $MgSO_4.7H_2O$) of analytical grade were used to assess the effect of heavy metals on the reproductive system *in vitro*. Stock solutions (1 M) were prepared by dissolving the metals in distilled water which was then stored at 4 ˚C until use. On the day of testing, 200, 100, 50, 25, 12.5, and 6.25 µM solutions of each metal was prepared in serum-free medium and used in the culture assay. These concentrations were selected because they are within ranges found in aquatic environments (WHO, 2004a; WHO, 2004b; WHO, 2004c).

5.3.5 Heavy metal and LH treatment of cells

Cells were seeded in 96-well tissue culture plates (Nunc, Serving Life Science, Denmark) at a volume of 25 µl per well. The cells were then treated with varying concentrations of each metal salt (200, 100, 50, 25, 12.5, and 6.25 µM) at a volume of 50 µl per well and incubated at 37 ˚C with 5 % CO_2 for 1 hour. Untreated cells were used as a control throughout the

study. The cells were thereafter incubated in the presence and absence of 10 mu LH per ml in serum free medium (25 µl per well) at 37 ˚C in 5 % CO_2 for 4 hours.

5.3.6 Cell viability

XTT assay was used to determine the effect of each heavy metal on cell viability. Cells were seeded in 96-well tissue culture plates (Nunc, Serving Life Science, Denmark), treated and incubated as previously described. At 1 hour incubation, XTT reagent mix was added as per manufacturer's instructions (Roche Diagnostics GmbH, Germany) and incubated at 37 ˚C with 5 % CO_2 for 4 hours. Formazan formation was then spectrophotometrically quantified at 492 nm (before and after 4 hour incubation) with a microtitre plate reader (Multiskan Ex, Thermo Electron Corporation).

5.3.7 Hormone production

Following the 4 hour incubation period, supernatants were assayed for testosterone and estradiol concentrations using commercially available enzyme-linked immunosorbant (ELISA) kits (DRG Instruments, GmbH, Germany) to assess the effect of manganese, copper, cadmium and magnesium on hormone production (testosterone and estradiol). The assays were performed as per manufacturer's instructions. The detection range of the testosterone and estradiol ELISA assays were between 0 – 16 ng/ml and 9.7 – 2000 pg/ml respectively.

5.3.8 Statistical analysis

For statistical analysis, SigmaStat 3.5 software package (Systat Software Inc., USA) was used. Each experiment was performed three times in triplicate and data was statistically analyzed via one-way analysis of variance (ANOVA) followed by the Tukey's test (differences when $P < 0.05$, were considered as statistically significant).

5.4 Results

5.4.1 Cell viability

Treatment of cells with cadmium and manganese (with/without LH-treatment) resulted in a significant decrease in cell viability (21 – 59 % (Figure 5.1B) and 34 – 84 % (Figure 5.1C) respectively) in a dose-dependent manner at all concentrations tested ($P < 0.05$). Cells treated with copper resulted in a significant decrease (dose-dependent) in cell viability at concentrations 25 – 200 µM (17 – 47 %) ($P < 0.05$) (without LH-treatment) and 6.25, 100 and 200 µM (23 – 48 %) ($P < 0.05$) (with LH-treatment) (Figure 5.1A) whereas a significant increase in cell viability (19 %) ($P < 0.05$) was observed in cells treated with magnesium (with/without LH-treatment) at a concentration of 25 µM (Figure 5.1D).

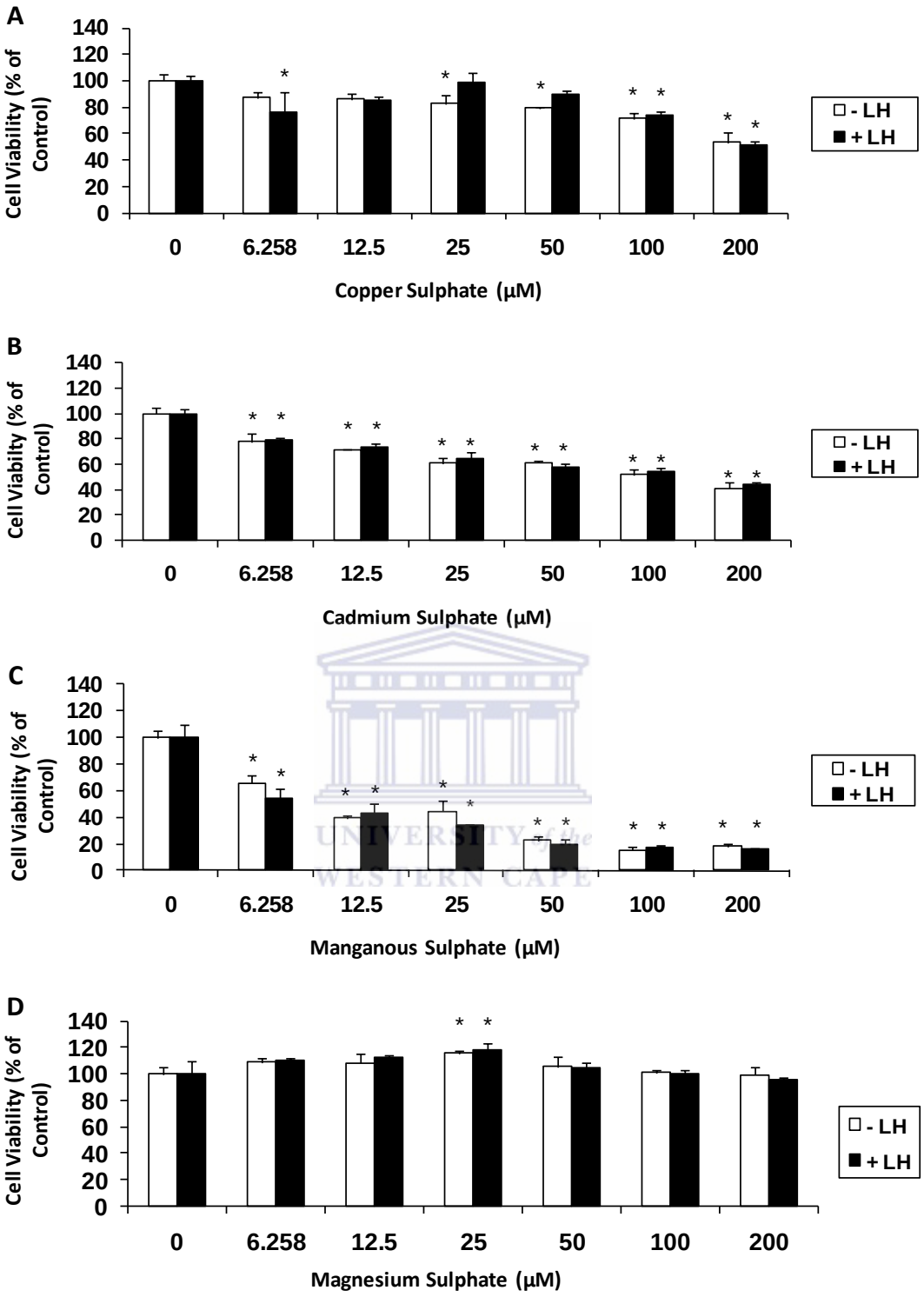

Figure 5.1 Effects of Cu (A), Cd (B), Mn (C) and Mg (D) on cell viability. Cells were treated with varying concentrations of each heavy metal salt (with/without LH-treatment). Values are means with corresponding SD bars. (* indicates $P < 0.05$ relative to the control; 0 µM – salt control)

5.4.2 Hormone production

Cadmium significantly decreased LH-induced testosterone production (49 – 95 %) in a dose-dependent manner at all concentrations tested ($P < 0.05$) (Figure 5.2B). Treatment of cells with copper (Figure 5.2A) and magnesium (Figure 5.2D) significantly decreased LH-induced testosterone production at concentrations 200 µM (33 %) and 25 µM (26 %), respectively ($P < 0.05$). No significant change in testosterone production was observed in cells treated with manganese (Figure 5.2C). Cells incubated in the absence of LH secreted low levels of testosterone and showed no significant effect. Estradiol production was undetectable at all concentrations tested (with/without LH-treatment) (data not shown).

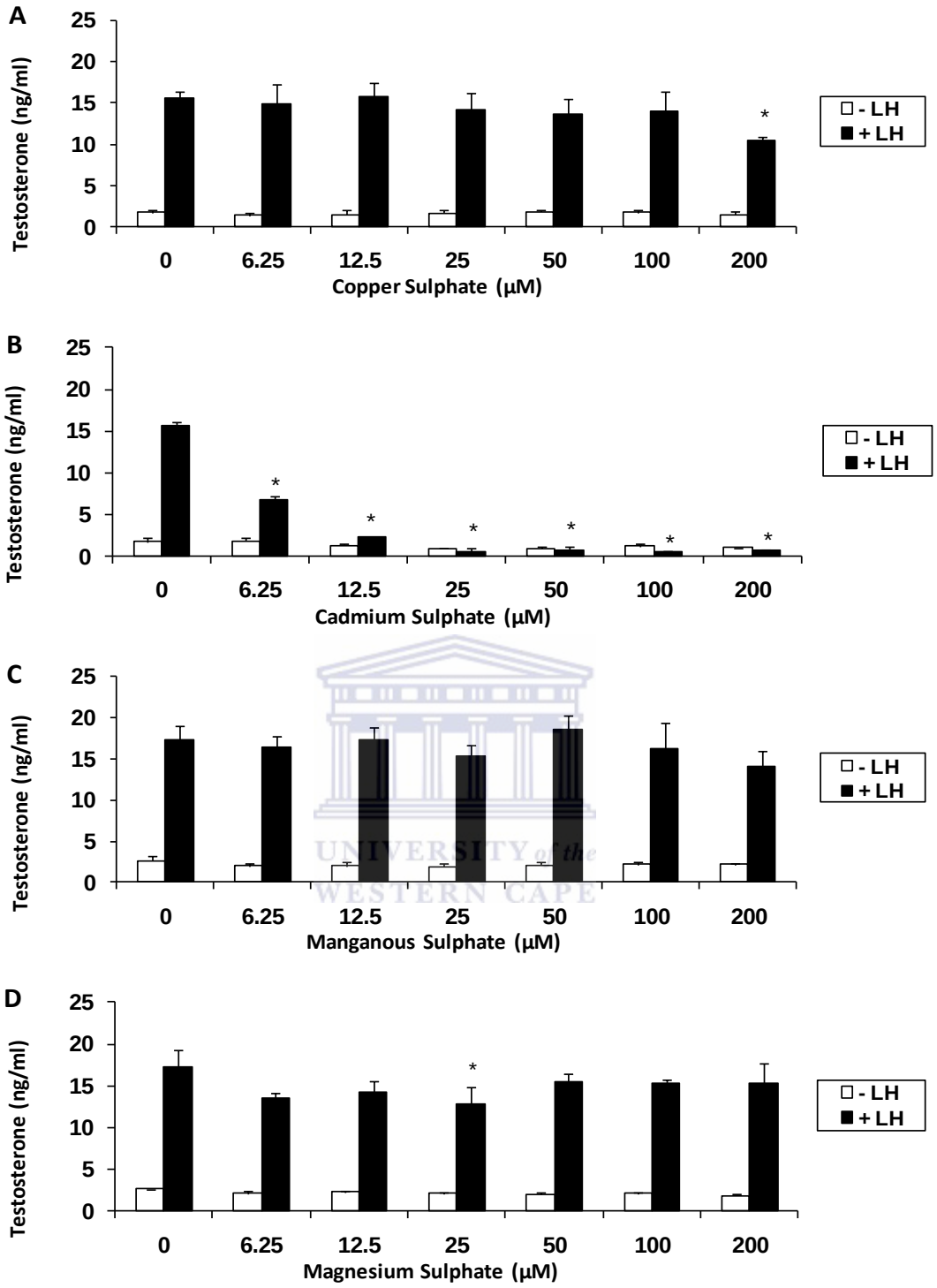

Figure 5.2 Effects of Cu (A), Cd (B), Mn (C) and Mg (D) on testosterone production (with/without LH-treatment). Cells were treated for 4 hours and supernatants were screened thereafter for testosterone. Values are means with corresponding SD bars. (* indicates $P < 0.05$ relative to the control; 0 µM – salt control)

5.5 Discussion

Normal functioning of the reproductive system is essential for normal sexual development and maintenance, behaviour, spermatogenesis, etc. (Mader, 2001; Seeley *et al*, 2003). Reproductive toxicity due to toxicant exposure (chemicals, pesticides, etc.) can occur as a result of direct or indirect effects on the reproductive system. Toxicity can occur at hypothalamic-pituitary level where toxicants can alter the function of these glands (hormone production – gonadotropin releasing hormone (GnRH), LH, follicle stimulating hormone (FSH)) (Fisher, 2004), or at a testicular level where toxicants can directly disrupt hormone production (testosterone production by Leydig cells – steroidogenesis) (Kumar *et al*, 2008; Murugesan *et al*, 2008) and cause cell death (Kim and Soh, 2009; Yang *et al*, 2003). The present study investigated the acute effect of Cu, Mn, Cd and Mg on the male reproductive system *in vitro* at the testicular level by using male Balb/c mice primary testicular cell cultures. Hormone production (testosterone, estradiol), and cell viability were endpoints that were investigated.

Under normal conditions, testosterone is produced in the testes by the interstitial cells of Leydig, which are found adjacent to the seminiferous tubules (Kumar *et al*, 2008). Testosterone production begins with the binding of LH (from the pituitary gland) to membrane-bound LH-receptors on Leydig cells which then results in a host of intracellular reactions which include signal transduction (formation of cyclic adenosine 3',5'-cyclic monophosphate (cAMP) and protein kinase A stimulation), cholesterol synthesis and transport to the inner mitochondrial membrane by steroid acute regulatory protein (StAR), and subsequent enzymatic conversions by several enzymes ultimately resulting in testosterone production. Changes in these biochemical pathways can result in a testosterone

imbalance which may then potentially result in reproductive toxicity (EPA, 2005). Adverse effects to the reproductive system due to toxicant exposure have been seen in both wildlife and humans such as demasculinization, decreased libido, infertility, congenital and developmental defects, testicular cancer, etc. (Gray Jr., 1998; Ong *et al*, 2002; Snyder, 2003).

In the present study, Cd was shown to decrease both testosterone and cell viability in a dose-dependent manner indicating that the reduction in testosterone levels is attributed to the reduction in cell viability. Also, testosterone production was completely inhibited at 25 µM while cell viability is still above 50 % at this concentration. These findings are in accordance with several previous studies where Cd was shown to cause testicular damage (Gunnarsson *et al*, 2003; Yang *et al*, 2003). Mechanisms which have been suggested to be involved in cadmium-induced testicular damage/injury include DNA damage (Yang *et al*, 2003), decreased LH-receptor mRNA expression (*in vivo*) (Gunnarsson *et al*, 2003), apoptosis due to reactive oxygen species (ROS) generation by Cd (Kim and Soh, 2009), etc.

Mn was shown to decrease cell viability but not testosterone production at all concentrations tested (200, 100, 50, 25, 12.5, and 6.25 µM) indicating that Mn is toxic to testicular cells but does not affect Leydig cells function (testosterone production). These findings are contrary to those found in a previous study where the authors found that cell viability was unaffected at concentrations lower than 1mM and testosterone production was significantly decreased (Cheng *et al*, 2003). The authors also found that the decrease in testosterone production was as a result of a reduction in StAR protein level, cytochrome $P450_{SCC}$ and 3ß-hydroxysteroid dehydrogenase (3ß-HSD) (enzymes involved in synthesis of testosterone). The decrease in cell viability may be due to Mn-induced oxidative stress.

A dose-dependent decrease in cell viability was seen in cells treated with copper but testosterone was only affected at the highest concentration tested. The copper-induced decrease in cell viability may be attributed to oxidative damage due to the fact that Cu is capable of ROS generation and DNA damage (Gaetke and Chow, 2003). The decrease in testosterone at 200 µM may be attributed to the decrease in cell viability which was seen at the same concentration tested. Treatment of cells with Mg did not affect cell viability and testosterone at all concentrations tested besides 25 µM. The decrease in testosterone production at this concentration may be attributed to the increase seen in cell viability which in turn may be due to increased metabolic activity. Cells treated without LH showed no significant effect in testosterone concentrations demonstrating that the metals do not affect testosterone production independently of LH. The decrease in cell viability observed in Mn, Cd and Cu-treated cells may be linked to oxidative damage due to the ROS-generating capability of these metals. These findings show that acute exposure of Cu, Mn and Cd to testicular cells is highly toxic. Further investigations into the exact mechanisms involved are warranted.

5.6 Conclusion

The data presented shows that manganese, cadmium and copper are highly toxic to testicular cells *in vitro* and therefore may potentially cause reproductive toxicity. Further studies are warranted to determine and clarify the exact mechanisms involved as well as *in vivo* studies to determine potential reproductive toxicity of each tested heavy metal.

5.7 References

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CHAPTER 6

Conclusion

EDCs pose a great risk to the health and quality of life of both humans and wildlife (Clotfelter *et al*, 2004). EDCs have been found in all major compartments of the environment, many of which are industrial chemicals (PCBs, BPA, etc.) pesticides (DDT, TCS, prochloraz, etc.), pharmaceuticals (EE2, KET, etc.) and phytoestrogens (genestein, quercetin, etc.). Several EDCs can cause adverse effects on the male reproductive system which include decreased sperm count, function and morphology, infertility, abnormal reproductive function and sexual behaviour as well as decreased libido, demasculinization and testicular cancer (Gray Jr., 1998; Ong *et al*, 2002; Snyder, 2003). These adverse effects, as a result of EDC exposure, have been associated with the ability of some EDCs to mimic reproductive hormones or to affect hormone synthesis and transportation (Clotfelter *et al*, 2004). There is an increased incidence of EDC contamination and exposure and it is thus imperative that screening tools be developed, validated and implemented to detect and monitor EDCs in the environment as well as to screen new substances with endocrine-disrupting capability.

In vivo methods are labour intensive, time-consuming, expensive, require large numbers of animals as well as highly specialized equipment and skills and alternative rapid screening tests for Tier 1 EDC monitoring are thus recommended. The EPA has recommended an *in vitro* minced testes method, above other *in vitro* and *in vivo* methods, as a suitable screening tool for EDC screening/monitoring due to low costs, reduced number of animals, use of

standard and basic equipment, the ability to screen large sample numbers with multiple endpoints as well as being quick and simple to perform and very sensitive (EPA, 2005).

In this study we have successfully employed an *in vitro* minced testes culture method to screen samples for male reproductive toxicity using hormone production and cell viability as endpoints. Following optimization, three plants, indigenous to southern Africa, used as traditional herbal remedies/medicines namely, *Tulbaghia violacea*, *Sutherlandia frutescens* and *Artemisia afra*, as well as four heavy metals (cadmium, manganese, copper and magnesium) used extensively in several industries, were successfully screened for their effect on testosterone production.

T. violacea, *S. frutescens* and *A. afra* were non-toxic to testicular cells. *T. violacea* displayed androgenic properties while *A. afra* has anti-androgenic properties at high concentrations. The androgenic properties of *T. violacea* suggest that it may potentially be used as a supplement to stimulate testosterone production.

Cadmium, manganese and copper are highly toxic to testicular cells *in vitro* and therefore may potentially cause reproductive toxicity and, thereby, endocrine disruption. These findings are especially worrisome as cadmium, manganese and copper are used extensively in many industries and the most common route of exposure to these metals is via contaminated drinking water sources. Also, manganese and copper are used extensively in fertilizers, fungicides and pesticides in the agricultural industry (Gimeno-García *et al*, 1996; Schneider *et al*, 2006) thereby contaminating food produce.

Our aim and objectives for this study, as shown by the results obtained, have been accomplished. The *in vitro* minced testes culture method is both rapid and sensitive as well as being inexpensive and simple to perform and is thus a valuable screening tool to detect and monitor EDCs in the environment. The method used in this study will now be employed in follow-up studies to screen food, water, and other environmental samples for potential endocrine-disrupting capability. This method will help us to detect, monitor and research environmental pollutants that may potentially cause adverse effects on animal and human health. It will, thereby, assist us to evaluate the current status of our environment so that we can develop risk assessment tools, management plans and countermeasures to protect the environment, wildlife and humans at risk.

6.1 References

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Appendix 1:

Ebrahim M, Pool EJ (2010). The effect of *Tulbaghia violacea* extracts on testosterone secretion by testicular cell cultures. Journal of Ethnopharmacology 132: 359-361.