

Investigation into potential endocrine disruptive effects of *Sceletium tortuosum*

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Declaration

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March 2018

What if I fall?
Oh, but darling what if you fly?

ABSTRACT

Depression has been recognised by the World Health Organisation (WHO) as the leading cause of disability, affecting an estimated 300 million people globally. To date antidepressants are prescribed as the first step in the treatment strategy. However, finding the appropriate antidepressant is often a lengthy process and is usually accompanied by side effects. A major and often unexpected side effect is reduced sexual function, which has been reported to aggravate depression and could possibly lead to poor compliance to medication.

Sceletium tortuosum is a native South African plant, which has exhibited both antidepressant and anxiolytic properties. Although the exact mechanism of action remains to be elucidated, there are currently two hypotheses which attempt to explain its mechanism of action. Firstly, it has been reported to act in the same manner as a selective serotonin reuptake inhibitor (SSRI), binding to the serotonin transporter and preventing the reuptake of serotonin (5-HT) and thus leading to an increase of 5-HT in the synaptic cleft. On the other hand, it has been reported to act as a monoamine releasing agent (MRA) which assists with the release of monoamine vesicles from the presynaptic neuron, also resulting in an increase of 5-HT in the synaptic cleft.

A strong correlation exists between depression and the hyper-activation of the hypothalamus-pituitary-adrenal (HPA) axis. On the other hand, antidepressants and MRA's have been associated with endocrine disruption. Although a human safety study has illustrated that *S.tortuosum* is safe for consumption, it was recently reported to decrease sex steroid hormone levels including androstenedione (A4) and testosterone (T) in the H295R cell line, but conclusive remarks could not be formulated. The objective of this thesis was to establish whether Trimesemine™ (Tri; a mesembrine-rich *S.tortuosum*) could adversely affect gonadal steroid synthesis.

HEK293 cells were transfected with 17 β HSD type 5 (17 β HSD₅) cDNA, resulting in the overexpression of the 17 β HSD₅ enzyme, which is responsible for the conversion of A4 to T. Previous studies within our group used Trimesemine™ at doses ranging from 0.0001 mg/ml to 1 mg/ml. For this study narrower ranges were chosen. The cells were

treated with TriTM at proven effective dose (0.01 mg/ml) and high (0.5 mg/ml) dose, the supernatant was removed after 24 hours and the samples were analysed by UPC²-MS/MS. Routinely euthanized mice were accessed immediately after the culling to obtain viable tissue for primary cultures. Testes were removed, decapsulated and a mixed culture was plated. After 24 hours the cells were treated with lower and high doses of TriTM, following another 24 hours the supernatant was removed and the samples were analysed with UPLC-MS/MS. Ovaries were collected, dissociated and plated. The mixed culture was allowed to stabilise for 24 hours. This was followed by treatment with TriTM at lower and high doses for a period of 24 hours, the supernatant was removed and the samples were analysed with UPLC-MS/MS.

At the lower dose TriTM did not appear to have any effect on A4, T and estradiol (E2). However, the high dose of TriTM appeared to decrease A4 (mixed testicular culture) and T (HEK293 cells), possibly via inhibition of the 3 β HSD or 17 β HSD enzymes. In contrast no there did not appear to be estrogenic effects.

In conclusion, low dose TriTM did not appear to exhibit endocrine disruptive properties *in vitro*. These doses are comparable to those recommended for consumer use. The adverse effect elicited by the high dose TriTM could be used as a marker for overdose, provided that basal levels of the hormones are known. These positive results indicates endocrine safety *in vitro*, but *in vivo* data is required for conclusive confirmation.

UITTREKSEL

Depressie word deur die Wêreldgesondheidsorganisasie (WHO) erken as die grootste oorsaak van ongeskiktheid, wat wêreldwyd sowat 300 miljoen mense raak.

Tot op datum word antidepressante as die eerste stap in die behandelingstrategie voorgeskryf. Om 'n toepaslike antidepressant te vind is dikwels 'n langdurige proses en gaan gewoonlik gepaard met nuwe-effekte. 'n Meestal onverwagte nuwe-effek is verminderde seksuele funksie, wat moontlik die depressie kan vererger, of die pasiënt 'n rede gee om hul medikasie minder as voorgeskryf te gebruik.

Sceletium tortuosum is 'n inheemse Suid-Afrikaanse plant, wat beide antidepressante en teen-angstigheids eienskappe het. Alhoewel die meganisme(s) van aksie van die plant nog nie geheel bekend is nie, is daar twee modelle wat dit probeer verduidelik. Dit word beweer dat *S. tortuosum* aan die serotonien reseptor bind om die heropname van serotonien te verhoed, en werk dus soos 'n selektiewe serotonien heropname inhibeerder (SSRI). Die tweede model beweer dat *S. tortuosum* help met die vrystelling van die monoamien neuroseinoordraer uit die presinaptiese neuron, en dus soos 'n monoamien vrystellingsagent (MRA) werk.

Daar is 'n sterk verband tussen depressie en die hiperaktivering van die hipotalamus-pituitêre-adrenale (HPA) as. Beide antidepressante en MRA's word geassosieer met endokriene ontwrigting. Hoewel 'n veiligheidstudie bevind het dat *S. tortuosum* veilig is vir menslike gebruik, is dit onlangs gerapporteer dat daar 'n moontlikheid is dat dit sekere steroïedhormone, soos androstenedioon (A4) en testosteroon (T), kan verminder, maar beslissende opmerkings kon nie geformuleer word nie. Die doel van hierdie proefskrif was om vas te stel of Trimesemine™ (Tri; mesembrine ryk *S. tortuosum*) moontlik 'n nadelige effek op steroïedsintese in die testis en/of ovarium het.

HEK293 selle is getransfekteer met 17 β HSD tipe 5 (17 β HSD₅) cDNA, wat lei tot die ooruitdrukking van die 17 β HSD₅ ensiem, wat verantwoordelik is vir die omskakeling van A4 na T. Die selle is voorafbehandel met Tri™ teen 'n bewese effektiewe dosis (0.01 mg / ml) en hoë (0.5 mg / ml) dosis. Na 24 uur was die supernatant verwyder en die monsters was ontleed deur gebruik te maak van UPC²-MS/MS. In 'n opvolgekperiment is primêre gemengde muistestis kulture opgestel. Testisse is verwyder, ontkapsel en 'n

gemengde kultuur was uitgeplaat. Na 24 uur is die selle behandel met lae en hoë dosisse TriTM. 24 uur later is die supernatant verwyder en die monsters was geanaliseer deur behulp van UPLC-MS / MS. Die ovaria is verwyder, fyngemaak en uitgeplaat. Na 24 uur is die selle behandel met effektiewe en hoë dosisse TriTM. 24 uur later is die supernatant verwyder en die monsters was geanaliseer deur behulp van UPC²-MS / MS.

By die laer dosis was daar geen nadelige effek op A4, T en estradiool (E2) nie. Die hoë dosis TriTM het egter vlakke van A4 (testikulêre gemengde kultuur) en T (HEK293 selle) verminder, moontlik deur inhibisie van 3 β HSD of 17 β HSD ensieme. Daar was geen verandering in die vlakke van E2 gesien nie.

Ten slotte blyk dit dat laer dosis TriTM nie *in vitro* endokriene ontwrigtende eienskappe vertoon nie. Hierdie dosisse is vergelykbaar met dié wat aanbeveel word vir verbruikersgebruik. Die nadelige effek wat deur verhoogde dosisse van TriTM verkry word, kan moontlik as 'n merker vir oordosis gebruik word, indien die basale vlakke van die hormone bekend is. Alhoewel die huidige studie dui op endokriene veiligheid *in vitro*, is *in vivo* data nodig vir voldoende bevestiging hiervan.

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LIST OF ABBREVIATIONS**General**

BSA	Bovine Serum Albumin
BZP	Benzyl piperazine
CAM	Complementary and Alternative Medicine
DMEM	Delbuco's Modified Eagle Medium
DMEM/F12	Delbuco's Modified Eagle Medium: Nutrient Mixture F12
FBS	Fetal Bovine Serum
HBSS	Hanks Balanced Salt Solution
HEK293	Human Embryonic Kidney cell line
HPA	Hypothalamus-pituitary-adrenal axis
HPG	Hypothalamus-pituitary-gonadal axis
IMM	Inner Mitochondrial Membrane
UPLC-MS/MS	Ultra Performance Liquid Chromatography-Mass Spectrometry
MAOI	Monoamine Oxidase Inhibitors
MDMA	3,4-methylenedioxy methamphetamine
NaSSA	Noradrenaline and serotonin-specific antidepressant
OMM	Outer Mitochondrial Membrane
PenStrep	Penicillin-Streptomycin
RFRP	FR amide related peptide
SERT	Serotonin Transporter
SNRI	Serotonin and Noradrenaline Reuptake Inhibitors
SSRI	Selective Serotonin Reuptake Inhibitors
ST	Sceletium tortuosum; <i>S.tortuosum</i>
TCA	Tricyclic Antidepressants
TFMPP	Trifluoromethylphenylpiperazine
Tri	Trimesemine™ a mesembrine-rich <i>S.tortuosum</i>
WHO	World Health Organisation

Enzymes

11OHA4	11 β -hydroxysteroid dehydrogenase
16OHPROG	6 α -hydroxyprogesterone
3 β HSD	3 β -hydroxysteroid dehydrogenase
17 β HSD ₅	17 β -hydroxysteroid dehydrogenase

AChE	Acetylcholinesterase
CB1	Cannabinoid receptor-1
CYP17	Cytochrome 17
CYP19	Cytochrome 19
CYP21	Cytochrome 21
HSD	Hydroxysteroid dehydrogenase
GABA	Gamma-amino butyric acid
MAO	Monoamine oxidase
P450 _{scc}	Cholesterol side chain cleavage
P450 _{aro}	Aromatase
PDE4	Phosphodiesterase-4
StAR	Steroidogenic Acute Regulatory Protein

Hormones and Steroids

5-HT	Serotonin
A4	Androstenedione
ALDO	Aldosterone
CORT	Corticosterone
DA	Dopamine
D2-T	De-eterated testosterone
D9-P4	De-eterated progesterone
DHEA	Dehydroepiandrosterone
E1	Esterone
E2	17 β -Estradiol
FSH	Follicle Stimulating Hormone
GnRH	Gonadotrophin Releasing Hormone
LH	Luteinising Hormone
NA	Noradrenaline
PREG	Pregnenolone
PROG	Progesterone
T	Testosterone

CHAPTER 1

INTRODUCTION

Depression is defined as an altered mental state, associated with feelings of low self-esteem, a despondent mood and reduced pleasure in daily activities (Bondy, 2002). At present the World Health Organisation (WHO) has recognised depression as the leading cause of disability, with an estimated 300 million sufferers globally.

The exact cause of depression remains to be elucidated, but currently there are biological, psychological and social models which attempt to explain the onset of the disorder. One of these models propose the dysregulation of the hypothalamus-pituitary-adrenal (HPA) axis (Roy and Campbell, 2013). A hyperactive HPA axis has been observed with the onset of depression (Swaab *et al.*, 2005; Chazittofi *et al.*, 2016). This hyperactivity results in an increased concentration of cortisol (CORT) within the body which has subsequently been associated with an increased susceptibility to develop depression. Another renowned model is the *monoamine deficiency model of depression*, this model suggests that depression arises as a result of a decrease or imbalance of monoamine neurotransmitters (dopamine (DA) and noradrenaline (NA) and serotonin (5-HT)) (Delgado, 2000).

Antidepressants are currently the first line of therapy to alleviate the depressive symptoms. There are several classes of antidepressants and each class operates through a different mechanism of action, but ultimately aim to improve the individual's mood. Selective serotonin reuptake inhibitors (SSRI) are usually prescribed as the antidepressant of choice, upon initial diagnosis of depression (Jakobsen *et al.*, 2017). SSRI increase the concentration of 5-HT within the synaptic cleft by preventing its re-uptake into the presynaptic neuron. Although antidepressants alleviate the depressed symptoms, finding the correct antidepressant is often a lengthy process which is accompanied by numerous side effects including nausea, dry skin and weight deviations. Sexual dysfunction is a major and often unexpected side effect, which could lead to poor self-esteem, relationship tension and feelings of inadequacy. This could exacerbate the condition and result in non-compliance of medication (Kennedy *et al.*, 1999).

Recently alternative and complementary therapies have gained popularity. Several natural remedies have been reported to relieve depression, such as St. John's wort and *Crocus sativus* (Saffron crocus) (Ernst, 2007). *Sceletium tortuosum*, a plant native to South Africa, has obtained interest for its antidepressant and anxiolytic properties (Smith, 2011; Nell *et al.*, 2013). Human and animal studies have indicated that it is safe for human use, with no reports of serious adverse side effects. Recently, a study by Smith and Swart (2016) observed potential undesired effects of Trimesemine™ (a mesembrine-rich *S.tortuosum*) on the steroidogenic pathway, as demonstrated in an adrenal cell culture model (H295R). This observation could indicate possible endocrine disruptive effect, which remains to be clarified.

As the endocrine system is critical for the regulation and overall function of the organism, understanding the adverse effects caused by compounds is essential. In human and animal studies it has been reported that antidepressants decreased testosterone (T) concentrations (Gregorian *et al.*, 2002). Specifically, in aqueous environments antidepressants such as fluoxetine, sertraline and citalopram had endocrine disruptive effects (Mennigen *et al.*, 2008; Mennigen *et al.*, 2010; Soga *et al.*, 2012). MRAs have also reportedly contributed to disruptions of the endocrine system. Owing to the mechanism of action (SSRI or MRA) of *S.tortuosum* as well as the indicative endocrine changes reported by Smith and Swart (2016), determining the effect in the context of gonadal steroid synthesis is essential.

CHAPTER 2

LITERATURE REVIEW

This literature review will offer a basic physiological overview in an attempt to describe the HPA and hypothalamus-pituitary-gonadal (HPG) axis as well as their interaction. In order to place this research in context, a more basic physiology will be followed by a discussion of what is known about the causes of depression, as well as the different treatment options. Furthermore, the negative side-effects associated with conventional treatments will also be highlighted. This will be followed by an overview of literature pertaining to a natural plant extract which shows promise in the context of depression.

2.1 Steroidogenesis

The process of synthesising steroids is known as steroidogenesis. This process occurs at several sites within the body including the adrenals, gonads, the brain and placenta (Tamaaki, 1972). Steroids synthesised within the body, are termed endogenous steroids and these are critical for the development and maintenance of life. Endogenous steroids are grouped into five classes namely: estrogens, progestins, androgens, glucocorticoids and mineralocorticoids (Giguère *et al.*, 1988; Miller, 2013). For the purpose of this review the focus centres around the production of sex steroids and the potential effects produced by endocrine disruptors on these steroids.

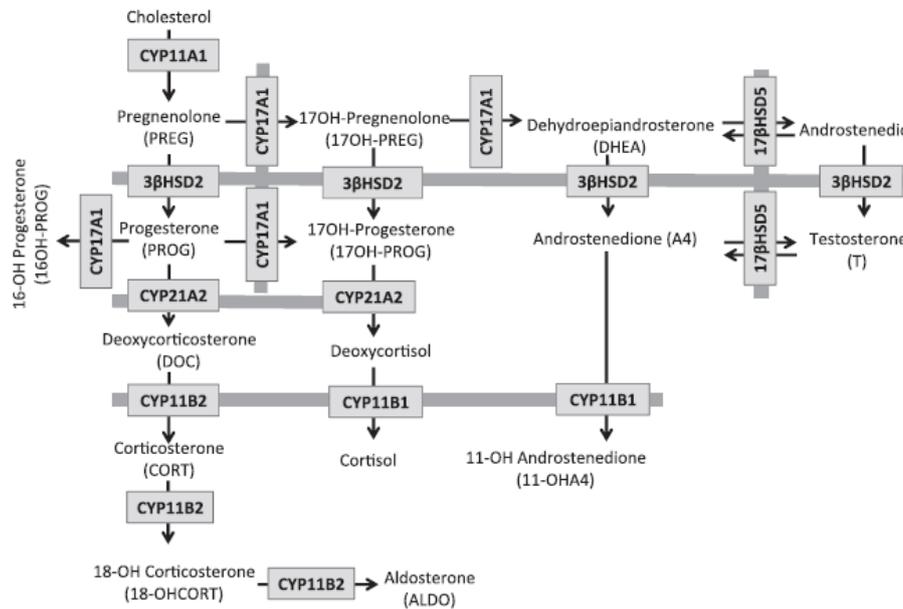
Steroids are not stored but synthesised as required by the body. Cholesterol is essential to the process of steroidogenesis, as it is the precursor for all steroids. Although some organs, such as the adrenals are capable of *de novo* synthesis, the preferred source of cholesterol is low density lipoproteins (Miller, 1998; Liu *et al.*, 2005). One of the rate limiting steps of steroidogenesis is the quantity of cholesterol. The process of steroid synthesis is governed by the steroidogenic acute regulatory protein (StAR), which is responsible for the movement of cholesterol from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM). Cholesterol will bind to StAR situated on the OMM, this interaction will promote a conformational change in StAR resulting in the release of cholesterol to the IMM (Miller, 2007; Rone *et al.*, 2009).

2.1.1 Adrenal Steroidogenesis

Adrenocorticotrophin hormone (ACTH) regulates adrenal steroidogenesis and this regulation occurs at three different time courses. Firstly, the long term effect (months or weeks) of ACTH can be observed by the process of hyperplasia and hypertrophy, thus governing the size of the adrenals. Secondly, over the course of days, ACTH promotes the expression of steroid enzymes, influencing the concentration of steroidogenic machinery. Thirdly, ACTH almost instantaneously stimulates the onset of steroid biosynthesis in response to the demands of the body (Grahame-Smith *et al.*, 1967; Miller, 2017). There are two enzymatic groups which are crucial for the production of steroid hormones, namely hydroxysteroid dehydrogenases (HSD) and cytochrome P450 (P450s) enzymes.

The adrenals have three distinct layers: the *zona glomerulosa*, *zona fasciculata* and *zona reticularis* and each zone is responsible for the production of different steroids: mineralocorticoids, glucocorticoids and gonadocorticoids respectively. As a result of this the zones require different enzymatic machinery. The *zona glomerulosa* contains aldosterone synthase (P450c11AS), which is required for the conversion 11-deoxycorticosterone (DOC) to aldosterone (ALDO). Cytochrome P450 11 β -hydroxylase (P450c11 β) is present in the *zona fasciculata* and it promotes glucocorticoid synthesis. Unique to the *zona reticularis* is the enzyme aromatase (P450aro), which aids the transition of androgens to estrogens (gonadocorticoids). The initial step in steroid synthesis requires P450 side chain cleavage (P450scc/CYP11A1), which is responsible for the conversion of cholesterol to pregnenolone (PREG). Therefore P450scc is present in all steroidogenic tissue (Hanukoglu, 1992; Jefcoate *et al.*, 1992). Another P450 enzyme which is critical for the steroid synthesis is Cytochrome P450 17 α -hydroxylase/17,-20-lyase (P450c17). This enzyme has dual activity and is the branch point in the synthesis of glucocorticoids or gonadocorticoids (Chung *et al.*, 1987; Brock and Waterman, 1999; Schloms, 2015) (as depicted in Fig.2.1).

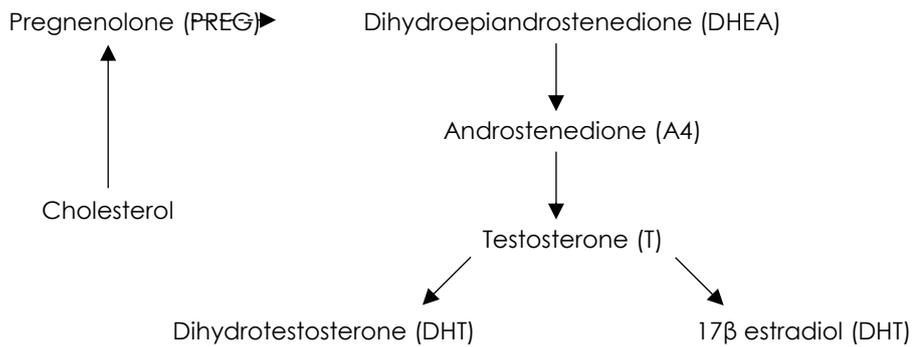
Figure. 2.1. Steroidogenic pathway present in the adrenal cortex (Smith and Swart, 2016)



2.1.2 Gonadal Steroid Synthesis

The Leydig cells are responsible for testosterone (T) production within the testes (Zirkin and Chen, 2000; Saez *et al.*, 1983). Unlike the adrenal cells, Leydig cells do not express sulfatases, preventing the conversion of dehydroepiandrosterone (DHEA) to dehydroepiandrosterone-sulfate (DHEA-S). The absence of these enzymes increases the concentration of substrate for T production. The final steps in androgen synthesis is the conversion of androstenedione (A4) to T and in some cases T is converted to its more active form dihydrotestosterone (DHT), via 17βHSD₃ or 17βHSD₅ and 5α-reductase enzymes respectively (Luu-The, 2001; Miller and Auchus, 2011)(Fig.2.2).

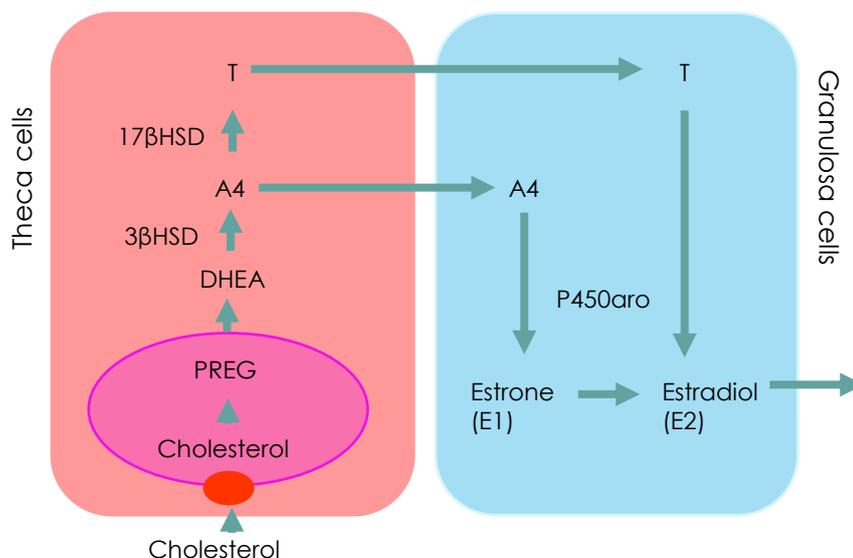
Fig.2.2. Steroidogenesis in the Leydig Cells



Steroid synthesis in the Leydig cells, along with the enzymes essential to the pathway. Steroidogenic acute regulatory protein (StAR); Cholesterol side chain cleavage (P450_{scc}); 3 β HSD; 17 β HSD; 5-ARD; P450_{aro}. The dotted lines represent the steroid pathway from PREG to DHEA (Adapted from Odermatt *et al.*, 2016)

Unlike the testes, steroidogenesis within the ovary occurs in a multistep manner, involving the theca and granulosa cells. Cholesterol will enter the theca cells, it will be converted to PREG and subsequently to A4 and T. These androgens then move into the granulosa cells, where they will be converted into estrogens, estrone (E1) and estradiol (E2), by means of aromatase enzyme (Payne and Hales, 2004) (Fig.2.3). E2 will be transported out of the granulosa cell and bind to an estrogen receptor of choice, resulting in a desired response.

Fig.2.3 The steroid synthesis in the ovaries.



Cholesterol moves into the mitochondria and is cleaved by P450_{scc}. In the Theca cells (pink block) cholesterol is converted to A4 or T, this then moves (green arrows) to the

granulosa cells (blue block). T could also leave the Theca cells at this point (purple arrow). In the granulosa cells A4 and T are converted to estrone (E1) and estradiol (E2) via P450_{aro}, and this hormone will move out of the granulosa cells (Adapted from Bloom *et al.*, 2016).

It is important to note that steroid production is under endocrine control and therefore the two main axes which are implicated will be briefly discussed.

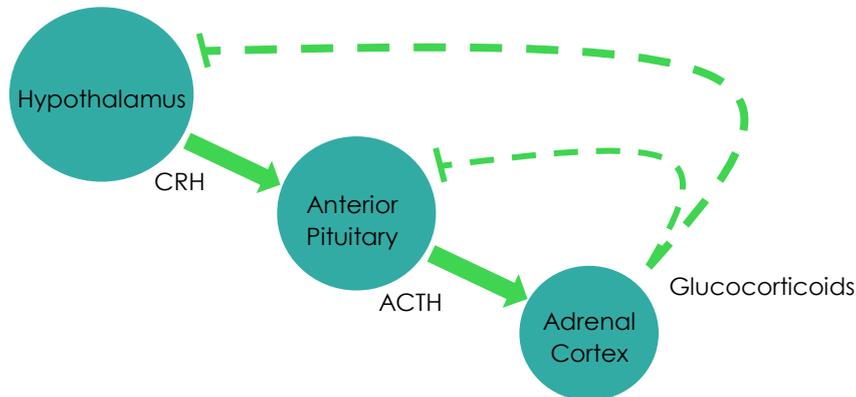
2.2 The hypothalamus-pituitary-adrenal (HPA) axis

Stress is defined as any stimulus that disrupts homeostasis of the organism, this disruption is countered by an appropriate response which was initiated by the HPA axis. In the late eighties and early to mid-nineties researchers aimed to clarify the definition of a stressor, the response it initiates and how the body adapts to the disruption. The well-known “fight or flight” term was coined by W. Cannon (Cannon, 1929; Johnson *et al.*, 1992) which suggests that the presence of a threat will result in an action of moving toward or away from the threat (Cannon, 1932). H. Selye (1950) elaborated on this model and named it the “*General Adaptation Syndrome*”. This model comprises of three phases: #1. *The Alarm Phase*; here the body recognises the stressor, activating the sympathetic nervous system (SNS) and resulting in a surge of CORT and epinephrine. #2. *The Phase of Resistance*; during this phase the body adapts to the stressor, allowing the organism to function optimally in the presence of the stressor. CORT and epinephrine will remain upregulated. #3. *The Phase of Exhaustion*; occurs under chronic stress conditions. During this phase the metabolic load can no longer be maintained as resources have been depleted, this phase is often accompanied by other disorders such as diabetes or cardiovascular disorders (Selye, 1950; Selye, 1951; Handa and Weiser, 2014).

Under normal physiological conditions a stimulus (eg. threat, pain, emotion) activates the HPA axis. This stimulus activates the paraventricular nuclei in the hypothalamus to secrete corticotrophin releasing hormone (CRH). Which in turn stimulates the anterior pituitary to secrete ACTH, resulting in the release of glucocorticoids (cortisol (humans) or corticosterone (rodents)) from the adrenal cortex (Anisman *et al.*, 1999). Simultaneously, the locus ceruleus–norepinephrine autonomic (LC-NE) system is activated. This promotes the secretion of norepinephrine (NE) into the blood which in turn will increase the heart rate, blood pressure and mental alertness (Johnson *et al.*, 1992; Tsigos and Chrousos, 2002; Handa and Weiser, 2014). In the absence of a stressor,

or after neutralisation of the stress stimulus, the body will re-vert back to baseline (Sherwood, 2008). This down-regulation of the HPA axis occurs through negative feedback, as the glucocorticoids act as inhibitors of the system (Tsigos and Chrousos, 2002; Hosseinichimeh *et al.*, 2015) (Fig.2.4).

Fig.2.4. HPA axis.



Solid lines present stimulation while the dashed line represent inhibition.

In altered physiologic conditions such as chronic stress, HPA dysregulation appears to ensue (Anisman *et al.*, 1999; Tsigos and Chrousos, 2002; Raone *et al.*, 2007). This dysregulation is often in the form of increased activity, resulting in excessive glucocorticoids, which in turn has widespread adverse effects (Johnson *et al.*, 1992).

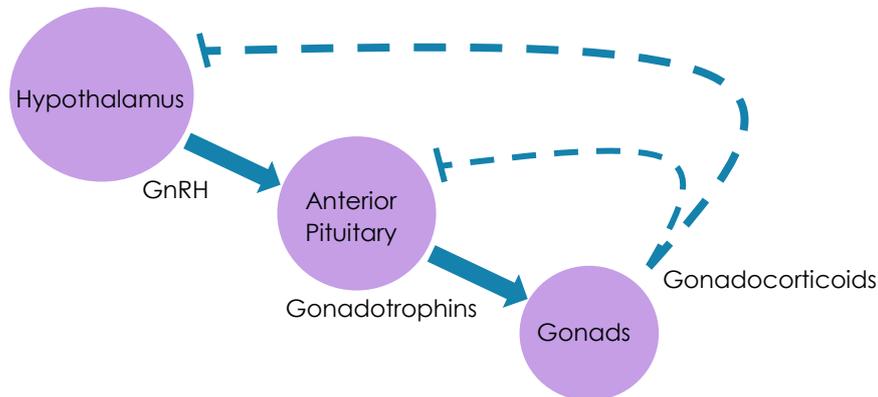
Interestingly, it has been observed that there are gender differences during the stress response and during the reproductive years these differences are more noticeable (Nolen-Hoeksema, 1987; Meller *et al.*, 200). Wang *et al.*, (2007) reported increased activation of the HPA-axis in men during performance tasks, while women showed a stronger activation during uncomfortable interpersonal encounters.

2.3 The hypothalamus-pituitary-gonadal (HPG) axis

Similar to the HPA, the HPG axis starts in the hypothalamus, with the pulsatile release of gonadotrophin releasing hormone (GnRH). GnRH stimulates gonadotropic cells in the anterior pituitary to release gonadotropins: follicle stimulating hormone (FSH) and luteinising hormone (LH). These gonadotropins are glycoprotein heterodimers, consisting of an α - and β -subunit. LH secretion is pulsatile and has been associated

with the pulsatile rhythm of GnRH. However, FSH secretion does not appear to have the same association with GnRH. These gonadotrophins will stimulate the release of sex steroids (estrogen, progesterone and T) from the gonads (ovary and testes) (Jin and Yang, 2014; Cross, 2017) (Fig.2.5).

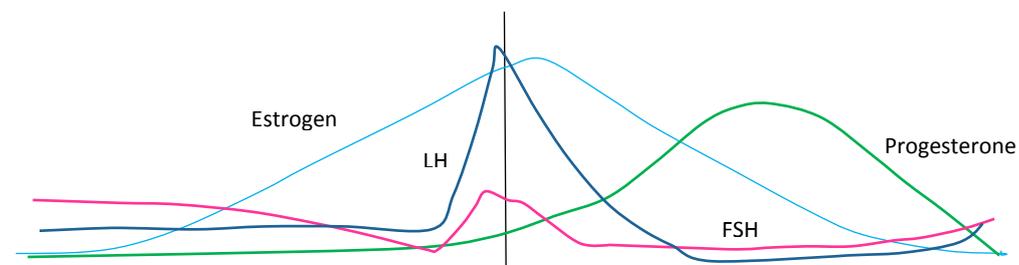
Fig.2.5. HPG axis.



Solid lines represent stimulation while the negative lines represent inhibition

These gonadotrophins have a particularly important role during the menstrual cycle. Throughout the cycle FSH and LH will fluctuate (Fig.2.6). The follicular phase is characterised by higher FSH concentrations, which will in turn stimulate the production of estrogen and promote follicular growth. Low levels of E2 from the developing follicle inhibits the release of LH but when E2 surpasses its inhibitory threshold, the inhibitory effect is lost and a surge in LH will ensue. This surge will initiate ovulation and an oocyte will be released. Lastly the luteal phase is characterised by high levels of progesterone (PROG), which has an inhibitory effect on LH and FSH. While the corpus luteum atrophies PROG decreases and LH and FSH will increase steadily (Hawkins and Matzuk, 2008; Sherwood, 2008; Cross, 2017).

Fig.2.6. Hormonal fluctuations during the menstrual cycle.



Ovulation is indicated by the black vertical line. Hormones are indicated as follows: progesterone (green), estrogen (blue), LH (brown), FSH (pink).

In males FSH and LH will also have reproductive roles. LH will bind to the receptors on the Leydig cells which will then promote the secretion of the T. FSH will bind to the Sertoli cells to provide an optimal conditions for spermatogenesis. Together, FSH and T allow for the maturation of sperm cells (Sherwood, 2008; Jin and Yang, 2014; Coss, 2017).

The HPG is also regulated through negative feedback, as the gonadal steroids will exert an inhibitory effect on GnRH, FSH and LH (Fig.2.5). This tight regulation between these hormones are critical for optimal reproductive function and thus any disruption in homeostasis of this system can lead to pathologies. For example, chronically high LH in women has been associated with the onset of polycystic ovary syndrome (PCOS) and high FSH has been linked to primary ovarian failure. While low T in men has been associated with infertility and poor semen quality (Sanderson, 2006; Cross, 2017; Sifakis *et al.*, 2017). There are various factors which could lead to HPG dysregulation, including stress, endocrine disruptors, physical abnormalities, surgery and certain medications (Hutchinson, 2002; Sanderson, 2006; Bloom *et al.*, 2016).

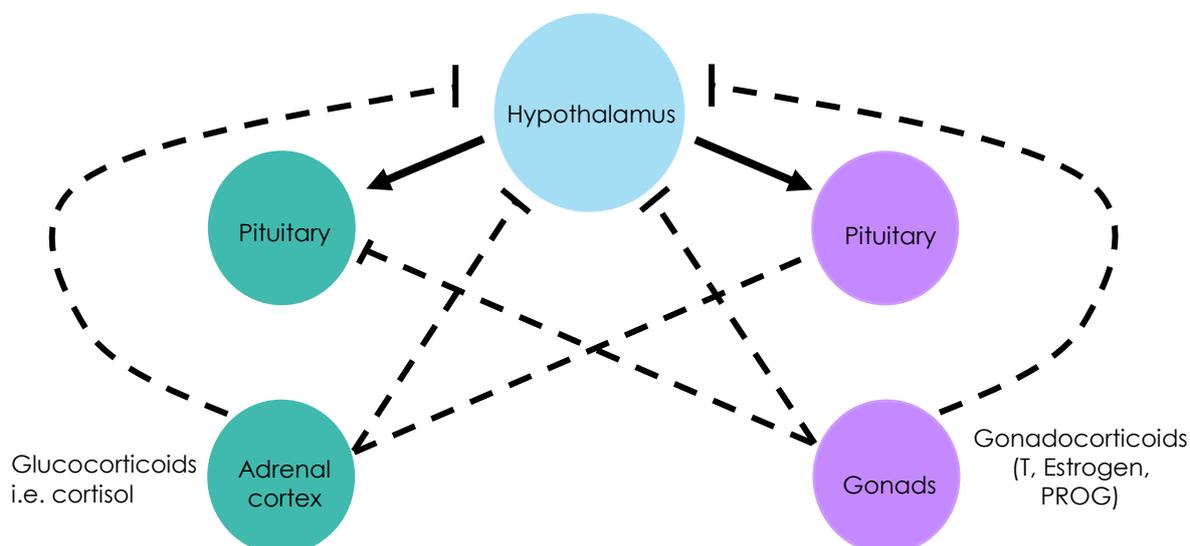
2.4 Functional connectivity of the HPA and HPG axes

Considering that both the HPA and HPG axes start within the hypothalamus and that both involve the pituitary gland, an interaction between the systems have been suggested (Roy and Campbell, 2013). During stress, the body will terminate activity of all non-essential process, such as reproduction (Rivier and Rivest, 1991; Angold *et al.*, 1999; Young and Korzun, 2002). Conversely, the HPG axis has been reported to inhibit the function of the HPA. It has been suggested that the end products of each axis is responsible for this inhibitory cross-talk (Viau, 2002)(Fig.2.7).

During adolescence there is a shift in the production of sex steroids from the adrenals to the gonads, this requires the activation of the HPG axis. During this period of life there is also increased pressure and conflict with oneself, parents and others, resulting in HPA activation. Therefore, it has been proposed that the HPA and HPG axis can function in unison, particularly during the period of adolescence (Johnson *et al.*, 2013; Green and McCormick, 2016). In a review by Rivier and Rivest (1991), a prominent relationship between stress and the activity of the HPG was highlighted. From their literature search they concluded that the effect of stress on the reproductive function

is present but the severity of this interaction is governed by factors such as the duration and intensity of stress. Viau *et al.*, (1999), reported that there is cross-talk between the HPA and HPG, via the end products CORT and T on hormones CRH and AVP.

Fig.2.7. Interaction between the HPA and HPG axes.



Interaction between the HPA and HPG axis (primary sites of cortisol and sex steroid production are indicated). The dashed line represents inhibition, while the solid lines are stimulation.

In the next section, we turn the attention to depression, with a discussion on the causes, as well as endocrine maladaptation which is commonly reported.

2.5 Depression

Globally 300 million individuals are reported to suffer from depression (WHO, 2017). Depression is classified as a mental disorder and is currently recognised by the World Health Organisation (WHO) as the leading cause of disability. When people appear to be saddened by certain life events they are carelessly called depressed. However it is important to recognise that depression is a serious and debilitating disorder that should be taken more seriously. Numerous individuals will experience symptoms of depression throughout their lives, however not all will be diagnosed with depression. In order to be diagnosed as depressed, individuals need to present five of the following nine symptoms over a two week period: despondent mood, considerable changes in weight or appetite, marked changes in sleeping pattern (insomnia or hypersomnia), diminished or excessive psychomotor activity, chronic exhaustion, poor

concentration, suicidal thoughts or tendencies and feelings of worthlessness (Bondy, 2002; Nestler *et al.*, 2002).

Depression is a complex disorder which varies between individuals in the onset, severity, symptoms and effective treatment. Although research has aimed to clarify potential causes for the disorder, to date a single causative factor has not been identified (Nestler *et al.*, 2002; WHO, 2017). Although the exact cause for depression remains unclear, a host of models have been formulated in an attempt to elucidate the onset of the disorder. These models have biological, psychological or social origins and each will briefly be discussed in the next section.

2.5.1 Potential biological aetiologies

2.5.1.1 HPA axis dysregulation

Depression has been strongly associated with the hyperactivity of the HPA axis. To establish HPA dysregulation CRH, ACTH and CORT concentrations are measured. The dexamethasone suppression test (DST) test is often used to determine the HPA response to cortisol. During this test Dexamethasone is administered at a low dose of 1 mg, following which the cortisol is evaluated to determine the response, if there is a decrease in CRH, ACTH and cortisol, the HPA axis is functioning correctly. However, if this is not the case a dose of 8 mg will be administered, to determine the reason for the excessive cortisol concentrations (Rybakowski *et al.*, 1999). Studies have reported mixed results with the DST. Some studies have reported an increase in CORT concentrations at awakening and in the evening (Vreeburg *et al.*, 2009). In support of this, studies have observed enlarged pituitary and adrenal glands (reviewed by Roy and Campbell, 2013). While others recognise there is an increased cortisol concentration at basal levels, but state that the stress response is dampened (Varghese and Brown, 2001; Burke *et al.*, 2005). It is important to note that the studies available in literature employ different methods and acquire samples at different times of the day using different stressors, offering a possible explanation for the different findings (Burke *et al.*, 2005; Powers *et al.*, 2016).

2.5.1.2 Monoamine Deficiency Model of Depression

Neurotransmitters are chemical messengers used by neurons for communication, to produce a desired effect. Those neurotransmitters which are relevant to depression are collectively known as monoamine neurotransmitters and include dopamine (DA),

noradrenaline (NA) and serotonin (5-HT). They are released from the presynaptic neuron into the synaptic cleft and then bind to receptors on the post-synaptic neuron (Sherwood, 2008). In the absence of a signal stimulating their release, they will return to the presynaptic neuron through endocytosis or undergo degradation by the monoamine oxidase (MAO) enzyme (Sherwood, 2008). Depressed individuals are reported to have lower concentrations of 5-HT relative to healthy controls. Therefore, current and developing therapies have aimed at increasing the amount of these neurotransmitters in an attempt to alleviate the despondent mood. To establish the importance of 5-HT in the context of depression tryptophan (5-HT precursor) depletion studies have been performed and reported an increase in depression-like symptoms. Additionally, the use of antidepressants elevate the concentration of these monoamines (Janowsky *et al.*, 1972; van Praag *et al.*, 1990; Delgado, 2000; Hirschfeld, 2000; Roy and Campbell, 2013).

Although these hold a relevancy to this thesis there are still more models which aim to explain the onset of this complex disorder. The *macrophage model of depression* proposes that the disorder is similar to an illness, and that inflammation present within the body results in the depressive symptoms (Maes *et al.*, 2009; Roy and Campbell, 2013). In post-mortem studies of depressed individuals structural abnormalities are common. It is suggested that these changes are brought about by the loss of neurogenesis and neuroplasticity. This contributes to atrophy within the brain leading to hippocampal volume loss and increasing the risk for the onset of depression (Bremner *et al.*, 2000). Often antidepressants increase neuronal growth factors such as brain derived neurotrophic factor (BDNF) and neuropeptide Y (NPY) which assist with these processes (Smith *et al.*, 1995; Drevets *et al.*, 2008; Roy and Campbell, 2013). The vulnerability for the onset of depression, is increased with genetic polymorphisms, specifically in the gene coding for the serotonin transporter promoter region (5-HTTLPR) (Nestler *et al.*, 2002).

2.5.1.3 Possible Psychosocial Factors

The emotional and mental aspects of depression are incorporated in the *psychological models* which include psychodynamic, behavioural and cognitive hypotheses. The *psychodynamic hypothesis* suggests the conscious and subconscious mind are constantly at conflict with each other and that an imbalance between these will result in depression. This model is used to explain the risk for

developing depression in those who have experienced early life trauma. It has been proposed that the manifestation of altered emotions and behaviour later in life occurs in response to poor emotional processing in children under traumatic (Nemade, 2014). The *behavioural hypothesis* states depression is learnt and that it can be unlearned. Furthermore, it also postulates the loss of the reward system, resulting in a subsequent loss of motivation and pleasure, which in turn increases the vulnerability for depression (Harmer *et al.*, 2004; Nemade, 2014). The *cognitive hypothesis* reasons that depressed individuals have a negative bias in their thought processing, observing the negatives and negating the positives – thus increasing the likelihood of developing a despondent mood which could lead to depression (Beck and Clarke, 2010; Nemade *et al.*, 2014).

2.5.2 Social factors

Social and cultural influences dictate which emotions are deemed acceptable for certain genders. Traditionally, women are tasked with child rearing and often manage a career, this could lead to her neglecting her own needs which will increase the vulnerability to develop depression (Abbey, 1991). Contrastingly, men have been raised to believe that a portrayal of their emotions shows reduced masculinity. The suppression of their emotions could result in chronic internal emotional turmoil which could increase their risk for the development of depression. This contributes to the poor diagnosis of depression in men, as these repressed emotions are regularly expressed as chronic pain, recurring disease or physical burdens e.g. headaches (Sewpaul, 1999; Nemade *et al.*, 2014).

2.5.3 Gonadal hormone maladaptation

As highlighted earlier, the HPA and HPG can interact with each other. It is important to remember that the end products each axis can result in the inhibition of its own axis, as well as the other axis (Fig.2.6). In the context of depression there is a strong association between the HPA and HPG axes. It has been observed that a hyperactive HPA axis, results in excessive CORT production, down-regulating the function of the HPG axis, consequently resulting in lowered T levels (Amiaz and Seidman, 2008).

Additionally, there seems to be a clear gender difference in the prevalence of depression, with women appearing to be more susceptible. However, women have been reported to seek professional help more readily than men, which could establish

the skewed gender difference (Möller-Leimkühler, 2002). Gonadal steroids exert an inhibitory effect on GnRH, and can also inhibit CRH. Early onset of depression is characterised by excessive levels of CORT and hyperactivity of the HPA axis, thus sharing traits associated with conditions such as amenorrhea and anorexia induced by chronic, severe exercise. In both these conditions individuals experience weight loss, increased CRH and disturbances of the menstrual cycle. The high CRH will also leads to inhibition of GnRH, which in turn will lead to decreased FSH, LH, estrogen and progesterone (PROG) (reviewed by Young and Korszun, 2002). Depression is often associated with excessive weight loss, which can contribute to reduced levels of E2 and PROG, along with a down regulation of the genes for the estrogen receptor α , progesterone receptor, FSH and LH, which in turn will have adverse reproductive implications (Zhou *et al.*, 2014).

Men with depression have been reported to have lowered T levels (Schweiger, 1999). This decrease in T with depression was also observed by McIntyre *et al.*, (2006). Although it remains unclear whether the decreased T could result in depressed or whether the depressive state reduces the T levels (Mikkelsen *et al.*, 2012).

In a study by Wang *et al.*, (2007) it was noted that using mental arithmetic tasks as the stress stimulus, men and women responded differently. Interestingly, men experienced an increase in salivary CORT while women did not. Giltay *et al.*, (2012) observed a positive correlation between T and psychological disorders in women but not men. Opposing this Welker *et al.*, (2014) found an association between CORT and T in men but failed to yield the same results for women. While Powers *et al.*, (2016) noted that depression in women was associated with lower CORT, while anxiety was associated with increased CORT. Furthermore, they observed increased CORT levels in depressed men.

In summary, from the literature, it is evident that males and females differ in their physiological response to stress. Perhaps in part due to the fact that the genders differ in terms of the relative stress vs. anxiety experienced in response to any particular stressor. In this context it is imperative to include both genders in research. Additionally, in the context of depression, maladaptation has been described in several different systems. This includes reduced reproductive function as a result of altered genes, reduced secretion of hormones and inhibition of the HPG axis (Viau, 2002). Despite this known effect of depression on the HPG axis, it seems that conventional western

medicine prescribed for depression largely fails to address the effect of depression on the reproductive system. When developing new treatment modalities it is important to consider not only, their capacity to alleviate the undesired deviations from homeostasis but also to ensure that the treatment does not aggravate any of these maladaptations. I provide a brief overview of commonly used antidepressants, in terms of their effect on the endocrine system.

2.6 Antidepressants

Presently, the first line of therapy for the treatment of depression is antidepressants. Considering the complexity of depression, finding the appropriate type of antidepressant and ideal dosage is often a lengthy process (O'Leary *et al.*, 2013). Although antidepressants produce immediate changes at a molecular level, it takes weeks to months of use for observable improvement in mood and well-being (Stahl, 1998).

There are five classes of commonly used antidepressant:

- *Monoamine Oxidase Inhibitors (MAOI)*: inhibit the action of monoamine oxidase, the enzyme which is responsible for the breakdown of monoamine neurotransmitters.
- *Noradrenergic and specific serotonergic antidepressant (NaSSA)*: responsible for blocking negative feedback effect on 5-HT and NA.
- *Serotonin and Noradrenaline Reuptake Inhibitors (SNRI)*: block the reuptake of both 5-HT as well as NA.
- *Serotonin Reuptake Inhibitors (SSRI)*: inhibit the serotonin transporter (SERT), thus preventing the reuptake of 5-HT in the presynaptic cell.
- *Tricyclic Antidepressants (TCA)*: prevent the reuptake of NA into the presynaptic cell (Werneke *et al.*, 2006).

First generation antidepressants such as TCA or MOAI, act on numerous sites, thus increasing the probability of side effects (Anderson, 1998). Newer antidepressant such as SSRI and SNRI focus on single monoaminergic neurotransmitters, reducing the incidence and severity of side effects, ultimately increasing the likelihood of drug compliance (Gregorian *et al.*, 2002). One of the most debilitating side effects which have been experienced after use of antidepressant medication, is diminished sexual

functioning (Safarinejad, 2008). The psychological stress associated with this perceived inadequacy can itself exacerbate the depressive state (Abbey, 1991). Thus, it is imperative to understand the underlying cause of this problem – which results from endocrine disruption.

2.7 Endocrine disruptors

Recently, investigations to determine the effects of endocrine disruptors has risen. Endocrine disruptors are defined as chemicals which lead to a dysregulation of hormone production, hinder hormonal actions or alter developmental processes which are under hormonal control (Sifakis *et al.*, 2017). Endocrine disruptors include the likes of pharmaceuticals, plastics and xeno-estrogens (herbicides, pesticides and fungicides) (Whitehead and Rice, 2006; Kabir *et al.*, 2015). For the purpose of this thesis, the focus will be on pharmaceutical endocrine disruptors.

Using the goldfish model, a commonly accepted model for this purpose, several antidepressants have demonstrated endocrine disruptive effects (Mennigen *et al.*, 2010). It was observed that, 54 µg/L fluoxetine (an SSRI and the active ingredient in Prozac), added directly to the water in the fish tank, inhibited both basal and pheromone-induced T production in sexually mature male goldfish (Mennigen *et al.*, 2010). Using female goldfish, the same group also reported an increase of central E2 levels, after fluoxetine was administered with an intraperitoneal injection (Mennigen *et al.*, 2008). However, this study also noted that the plasma levels of E2 were reduced, leading to the conclusion that increased central E2 production may down-regulate the HPG axis.

However this is, contradictory to results which was reported for mianserin a TCA antidepressant. Mianserin had estrogenic effects both centrally and in the gonads of both male and female zebrafish (Van der Ven *et al.*, 2006). Incidentally, in this study, the estrogenic effects were paralleled by reduced T levels, which again highlights an undesired effect on androgen production. Very recently, Islin *et al.*, (2017) also reported similar estrogen increasing and T decreasing effects for some SNRI antidepressants (duloxetine, venlafaxine and tramadol) in zebrafish. Their results indicated that doses above 20 µM produced inhibitory effects for the CYP17 and CYP21 enzymes – resulting in reduced T, A4 and 11βhydroxy-androstenedione

(11 β OHA4) – but appeared to increase the activity of the CYP19 enzyme (aromatase) thus increasing estrogen.

Similar endocrine disruptive effects have been illustrated using rodent models. For example, chronic administration (21 and 28 days) of Citalopram – another SSRI – has been shown to reduce the RF-amide related peptide (RFRP) neuronal numbers in the dorsomedial hypothalamus, as well as fiber projections to the preoptic-GnRH neurons (Soga *et al.*, 2010). Of specific concern for this particular SSRI, is the potential for transgenerational propagation of endocrine disruption. This potential adverse effect has been illustrated in male mice which were exposed to Citalopram early in life (postnatal days 8-21, simulating mother-to-breastfeeding offspring transfer (Soga *et al.*, 2012). Apart from increased anxiety-behaviour, mice also exhibited decreased androgen receptor levels, which could explain the altered sexual behaviour reported in these animals. Most recently, these results were also confirmed for the six most widely used SSRI drugs (fluoxetine, paroxetine, citalopram, escitalopram, sertraline and fluvoxamine) in a human adrenocortical cell line (H295R) (Hansen *et al.*, 2017), again implicating CYP17 inhibition.

In terms of the newly developed NaSSA drugs, I could find no information in the context of endocrine disruption. Given the impact on reproductive dysfunction, this remains to be investigated.

Although not technically classified as antidepressant medicine, it is known that many individuals suffering from depression may resort to consumption of illegal products, such as the MRAs, amphetamine and MDMA (Ecstasy) (Boyd, 1993; Huizink *et al.*, 2006). Given the effect of MRAs to increase 5-HT, DA or NA signalling, it is perhaps not unexpected that in terms of endocrine disruption, similar undesirable effects have been recorded for these drugs (Barenys *et al.*, 2009; Barenys *et al.*, 2010). Amphetamine treatment (which exerts its action through the dopaminergic system) has been shown to reduce T production in rat testicular interstitial cells by inhibition of the 3 β HSD, CYP17 and 17 β HSD enzymes (Tsai *et al.*, 1997). Similarly, treatment with MDMA (which functions via the serotonergic system) caused testicular degeneration in rats (Barenys *et al.*, 2009), suggesting a direct inhibitory effect on T as outcome. In addition, a central inhibitory effect on the HPG axis was also reported for MDMA, which again resulted in decreased T levels in male rats (Dickerson *et al.*, 2008). Although, research in females was not available for MDMA or amphetamine

specifically in this context, other so-called “party pills” - benzylpiperazine (BZP) and trifluoromethylphenylpiperazine (TFMPP), which function similarly to MDMA – have been shown to have estrogenic effects in female rodents (Min *et al.*, 2012).

Futhermore, given the consumer bias toward using natural products, phytoestrogens is another group of compounds that should perhaps be mentioned here. Phytoestrogens are compounds present in plants, that have a similar structure to that of estrogen (Mense *et al.*, 2008). There is still considerable controversy regarding the safety of these compounds (Whitehead and Rice, 2006; Mense *et al.*, 2008; Velentzis *et al.*, 2008), some arguing it has anti-cancer effects (Ingram *et al.*, 1997) while others postulate adverse effects, including risk of estrogen-related disorders owing to the increase of circulating estrogen levels (Sakamoto *et al.*, 2010; Nielsen, *et al.*, 2015).

The rise in depression in modern societies is reflected with increased antidepressant consumption and although it prompted the development of new antidepressants, no evidence is available to suggest that these newer drugs will have less endocrine disruptive effects. This, as well as the high cost of conventional medication, has encouraged a shift toward the use of alternative therapies, although these treatments also have the potential for endocrine disruption. For this reason, it has become essential to determine the effect of any treatment modality – whether pharmaceutical or nutraceutical – on the endocrine system.

One of these alternative regimes include the treatment with exogenous gonadal steroids. Schmidt *et al.*, (1999) suggested that depressed peri-menopausal women reported improved mood when receiving oral 17 β -Estradiol supplementation. Similarly, Galea *et al.*, (2002) reported that treatment with E2 improves the behaviour of an animal model for post-partum depression. From this data, it seems that hormone therapy may be a consideration as treatment for depression. However, hormone therapy is known to have other side-effects, such as increased risk for some types of cancer and stroke (Million Women Study Collaborators, 2003; Vigen, 2013). Another option for treatment entails a more holistic approach, such as that set out by Sarris (2011). Sarris proposes that a treatment strategy based on the *non-pharmacological antidepressant-lifestyle-psychological-social model*, could yield improved mood, as it aims to improve the general well-being. However, this approach may have poor compliance, as it is not always possible to remove stress from the modern lifestyle. Therefore, a third, more feasible option, is to turn to natural remedies with potential

antidepressant activities, but hopefully without the undesired endocrine disruptive effects reported for many pharmaceutical antidepressants.

Over the past few years there has been a growing preference for complementary and alternative medicine (CAM). Additionally, traditional healers are often the primary healthcare practitioners for those living in rural areas, specifically in developing countries as the rural communities are not in close proximity to clinics or hospitals (Ernst, 2000; Coulter and Willis, 2004; Sato, 2011; Goroll, 2014; Burns and Tomita, 2015). The three primary reasons for the preference of complementary medicine include: the dissatisfaction with conventional treatment strategies, an increased need to be in control of their health care choices and lastly CAM often aligns with patient's perceptions, cultural and religious belief systems. Under closer investigation, it was noted that the latter offered convincing support for the reason why people make use of CAM (Astin, 1998; Sorsdahl *et al.*, 2009).

Although, CAM offers numerous beneficial effects, it is important that these natural remedies undergo the same efficacy, efficiency and safety evaluations as pharmaceutical medications (Firenzuoli and Gori, 2007; Sato, 2011; Goroll, 2014).

2.8 *Sceletium tortuosum*

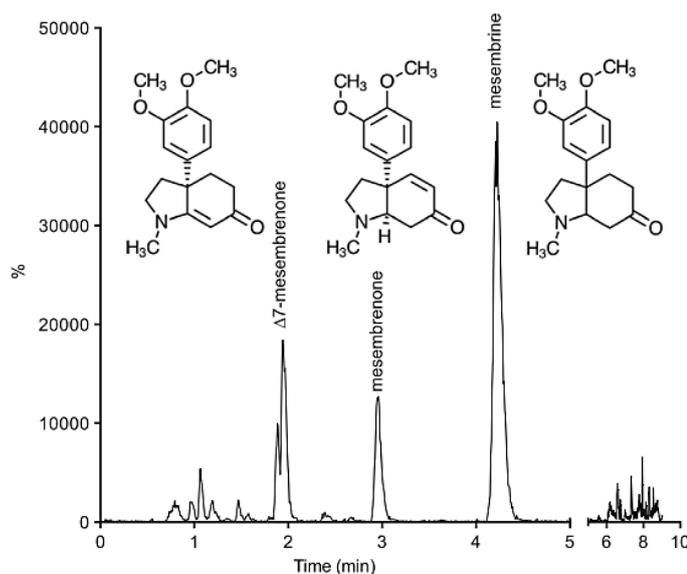
In the context of anxiety and depression, *Sceletium* sp. has gained much interest. Various species of *Sceletium* alkaloids have been extracted and studied (Shamma and Rodriguez, 1968; Arndt and Kruger, 1970). *Sceletium tortuosum* is part of the *Mesembryanthemaceae* family and is present natively in the Western and Northern Cape of South Africa. The plant received its name from its phenotypic characteristics: *Sceletus* (skeleton-like) owing to its veiny leaves and *tortuosum*, which means twisted (Gericke and Viljoen, 2008).

Anecdotally, the South African native Khoi and Hottentot populations used this plant for medicinal purposes to alleviate a variety of common ailments, such as abdominal pain, toothache, relief of hunger and pain. In infants, it was used to alleviate colic and as a calming agent (Gericke and Viljoen, 2008; Harvey, 2011). It was normally chewed (this route of administration resulted in its nickname 'Kougoed', while 'Kanna' was its folk name) but can also be consumed in the form of tea or snuff. Recently it has received more interest as a potential anxiolytic or anti-depressant as a result of its

psychoactive nature. Indeed, anecdotal use describes “intoxication” as an indicator of use (Gericke and Viljoen, 2008), suggesting that the plant may have mood-altering effects.

The active compounds to which the mood-altering effects of *S. tortuosum* are ascribed, are known as alkaloids. There are four primary alkaloids within the plant namely: mesembrine ((3*aS*,7*aS*)-3*a*-(3,4-dimethoxyphenyl)-1-methylhexahydro-1*H*-indol-6(2*H*)-one), mesembrenone ((3*aR*,7*aS*)-3*a*-(3,4-dimethoxyphenyl)-1-methyl-3,3*a*,7,7*a*-tetrahydro-1*H*-indol-6(2*H*)-one), mesembrenol ((3*aR*,6*S*,7*aS*)-3*a*-(3,4-dimethoxyphenyl)-1-methyl-2,3,3*a*,6,7,7*a*-hexahydro-1*H*-indol-6-ol) and mesembranol ((3*aS*,6*R*,7*aS*)-3*a*-(3,4-dimethoxyphenyl)-1-methyloctahydro-1*H*-indol-6-ol) (Krstenansky, 2017) (Fig.2.8). Mesembrenone content is the most variable between plants, but mesembrine is always the major alkaloid (Shikanga *et al.*, 2012).

Fig.2.8 Active compounds in Trimesemine (mesembrine-rich *S. tortuosum*).



UPLC-MS chromatographic separation of alkaloids in TRI. Compounds eluted as follows: Δ7-mesembrenone, mesembrenone, and mesembrine (Coetzee *et al.*, 2016).

Although, animal studies have been carried out to determine the effects of *S. tortuosum*, limited pharmacological and clinical studies have been conducted. Even though there is evidence supporting the beneficial effects of this plant, it remains essential to determine the adverse effects. Knowing the beneficial and detrimental

effects of the product is will allow the consumer to make an informed decision, prior to using the treatment.

A number of early studies have proposed that *S. tortuosum* acts as both a SSRI as well as a phosphodiesterase type 4 (PDE4) inhibitor (Harvey *et al.*, 2011). Zembrin, a commercially recognised *S. tortuosum* product, was pharmacologically processed to determine possible receptor binding. From this it was suggested that the whole product has a significant inhibitory capacity for the serotonin (5HT) transporter, GABA receptor, δ_2 -opioid receptor, μ -opioid receptor, cholecystinin-1. Following this they aimed to establish whether individual alkaloids could be responsible for this inhibition. From this it was observed that significant inhibition was present for SERT in the presence of mesembrine (Harvey *et al.*, 2011). Furthermore, they attributed the PDE4 inhibitory activity to mesembrenone. This inhibitory effect of *S. tortuosum* on the 5HT transporter, offers support to *S. tortuosum* use as an antidepressant.

In support of this hypothesis, another study was conducted using different doses of *S. tortuosum* (10 mg/kg and 80 mg/kg) which was administered intraperitoneally. The rodent forced swim test, a well-recognised model with which to determine the efficacy of an antidepressant, indicated an antidepressant effect with only the low dose (10 mg/kg) treatment (Schell, 2014). Somewhat similar to this, doses of 5 and 10 mg/kg of *S. tortuosum* (Trimesemine™) was employed to elucidate psychological effects in rats making use of the elevated plus maze (Smith, 2011). Results indicated that there was a decrease in CORT and self-soothing behaviour with the low (5 mg/kg) dose. Although the high (10 mg/kg) dose resulted in some undesirable reactions, this could be ascribed to the fact that the extract used was much cruder when compared to which was used prior to this study.

A novel therapeutic avenue which may have relevance is cannabinoid receptor 1 (CB1). This receptor has been reported to play a role in emotional processing and is co-localised with 5HT. Studies have reported that CB1 inhibition could potentially result in an antidepressant effect (Akirav, 2011; Pietrzak *et al.*, 2014). Research by Lubbe *et al.*, (2010) observed that whole *S. tortuosum* extract (0.037 mg/ml), and not individual compounds, have an inhibitory action on the CB1 receptor, which could offer an alternative, or additional, pathway by which to explain the antidepressant effects which have been reported (Loria *et al.*, 2014).

In contrast to earlier suggestions of a SSRI action for *S. tortuosum*, Coetzee *et al.*, (2015) suggested that *S. tortuosum* is primarily a MRA, with a secondary SSRI function. In addition to the MRA property this study also reported mild inhibitory effect on the monoamine oxidase A enzyme. This activity could offer another explanation to the possible antidepressant effects. It is important to note that this study made use of Trimesemine™, mesembrine-rich alkaloid, while other extracts are normally low in mesembrine. This difference in the mesembrine content could explain why other studies have not reported the monoamine release activity. In addition, both Loria *et al.*, (2014) and Coetzee *et al.*, (2016) suggested a minimal inhibition of acetylcholinesterase (AChE) activity.

There is limited information regarding the endocrine effects of the plant. As mentioned before, this information is essential, as it affects consumer safety and efficacy. Smith and Swart (2016) comprehensively reported on the effects of high-mesembrine *S. tortuosum* (Trimesemine™) extract on the glucocorticoid, mineralocorticoid and sex steroid pathways in an *in vitro* model using human adrenocortical (H295R) cells. It was observed that there was a dose-dependent decrease in both basal and stimulated production of the following steroids: cortisol, corticosterone (CORT), aldosterone (ALDO), 17-hydroxyprogesterone (17OHPROG), PROG, 16 α -hydroxyprogesterone (16OHPROG), A4, T, 11OHA4, while PREG was increased. These changes were reported at high concentrations (1 mg/ml), with the exception of A4 and T, which reported decreases at doses ranging between 0.0001 to 1 mg/ml. Therefore it is possible that *S. tortuosum* exerts its inhibitory effects through the CYP17 or 3 β HSD enzymes (Smith and Swart, 2016). Data obtained for androgens using H295R cells cannot conclusively determine the effect on gonadal steroidogenesis, as there are slight differences between the cell types.

Toxicology is a major factor which must be accounted for when assessing a potential therapeutic agent. Fortunately, several toxicology studies have been conducted and none have reported severe adverse effects related to the product (Zembrim at 8mg and 25mg, human subjects) (Nell *et al.*, 2013). Therefore, given the potential of this seemingly safe drug, it is imperative to determine whether potential undesired side-effects as normally associated with anti-depressant treatment, is associated with this plant.

2.9 Summary

To summarise, it is evident that there is interaction between the HPA and HPG axis, furthermore in the context of depression, there appears to be an inhibitory effect on the HPG axis via the HPA axis. Antidepressants are currently the first line of therapy for the treatment of depression, but these drugs have been associated with the dysregulation of sexual function. For this reason people have looked to alternative therapies, which can alleviate the symptoms, but without the cost of sexual dysfunction. One of the compounds of interest in *S. tortuosum*, which has reported limited side effect in human and animal studies. As sexual dysfunction results from endocrine disruption, it is essential to determine the effect on the endocrine system when investigating new treatment modalities

2.10 Hypothesis and aims

For this project it was hypothesised that the proven effective anti-stress dose of Trimesemine™ (a mesembrine-rich *S.tortuosum*) would not have undesired endocrine disruptive effects in the context of gonadal steroidogenesis.

In order to test this hypothesis, the following aims were formulated:

- The investigation of potential inhibitory effects of Trimesemine™ on selected p450 enzymes required for T production, in a human kidney cell culture transfected with 17 β HSD
- To investigate gonadal steroidogenesis in the presence of Trimesemine™ for both genders, using primary mixed testis and ovarian cell cultures

CHAPTER 3

METHODOLOGY

This thesis will present experimental methods and data on three different experimental protocols. Firstly, potential inhibitory effects of *S. tortuosum* on T synthesis was investigated in human kidney cells transfected with 17 β HSD. This data was followed up on in a study using a rat primary mixed testicular cell culture. Lastly, given the gender differences observed in the literature, a similar set of experiments was performed in rat primary mixed ovarian cell culture. Unique to this project was the two primary cultures, as this has not been carried out by our group before, although we have conducted research on several other cell culture models.

As per standard protocol, experiments were run in triplicate and repeated at least three times (i.e. for primary cultures, different animals were also used).

3.1 Experiment 1: Transfection of HEK293

The aim of this experiment was to transiently transfect HEK293 cells with the 17 β HSD enzyme which is responsible for the conversion of A4 to T.

3.1.1 Cell Line and Vectors

HEK293 cells as well as freezer stocks of *E.coli* (strain JM109) containing the plasmid vectors pCINeo or pcDNA3/17 β HSD₅ were kindly donated by Prof AC Swart (Dept. Biochemistry, Stellenbosch University). The passage number for this experiment ranged from 11 to 14.

3.1.2. Purification of Plasmid DNA

100 μ l of starter cultures of *E.coli* containing the plasmid vectors pCINeo or pcDNA3/17 β HSD₅ were added to 100ml of Lysogeny broth (LB) media containing 0.1% Ampicillin. The culture was homogenised on a shaking incubator (Innova, New Brunswick, Canada) at 215 rpm and 37 °C for 18 hours. The plasmid cDNA (human) was purified with NucleoBond® Xtra Plasmid Purification as advised by the manufacturer and stored at -20 °C. The DNA plasmid concentrations and 260/280 ratios were determined through UV spectrophotometry (Cary60 UV-Vis by Agilent technologies; Santa Clara, CA, USA). Restriction enzyme digests using BamH1 and

EcoRI, were used to determine the presence of the gene. Purified DNA was stored at -20 °C until they were required.

3.1.3 Transfection of HEK293 cells

HEK293 cells were cultured at 95% CO₂ and 5% N₂, at 37°C in supplemented DMEM (10% FBS and 1% PenStrep added) (Thermofisher; #41966052, #10499044 and #15140122 respectively) and passaged thrice, after which the cells were seeded in 24 well plates (Nest, #) at a density of 2 x 10⁵ cells per well in 500µl media. After 24 hours the media was removed and replaced with un-supplemented DMEM, as supplementation has been reported to interfere with transfection success. The cells were transiently transfected with 0.5 µg plasmid cDNA per well using X-tremeGene HP DNA Transfection Reagent (Roche; #0636654600). The pCINeo vector without the plasmid cDNA was used as a negative control for transfection. The cells were incubated for 48 hours to allow for transfection to occur. The transfection media was removed and the treatment interventions were applied for 12 hours. Treatment groups are described in table 3.1. Briefly, 2 doses of Trimesemine™ extract (batch #DV SCTR3:E 725076, Verve Dynamics, Somerset West, South Africa) were used in the experiment, the high dose at 0.5mg/ml and the low dose at 0.01mg/ml, both in DMEM (un-supplemented). Here it is important to state that the mesembrine-rich *S.tortuosum* (Trimesemine™) was extracted using proprietary methods. These dosages were decided upon as previous studies conducted by our group used a range of 0.0001 mg/ml to 1 mg/ml, thus the dosages used for this range offer a narrower range. The A4 (dissolved in absolute ethanol) was used at a concentration of 1µM for all experiments. Each well contained 600µl total volume, the balance was achieved using supplemented DMEM. After 12 hours media was collected in glass vials (Lasec; #G3TUB029Z-013100) and stored at 4°C for steroid extractions. For a detailed method description refer to addendum A.

Table 3.1. Experimental layout for the HEK293 cell experiment.

Transfected	+ Trimesemine™ (Tri)	- Trimesemine™ (Tri)
Untransfected	DMEM + Tri + A4	DMEM + A4
pCINeo	DMEM + Tri + A4	DMEM + A4
17βHSD5	DMEM + Tri(high) + A4	DMEM + A4
17βHSD5	DMEM + Tri(low) + A4	DMEM + A4

3.1.4 LC-MS/MS analysis of steroid metabolites

A steroid mix containing A4 and T was used to construct a standard steroid range (0, 0.5, 1, 5, 10, 25, 50, 100 and 250 ng/ml) which was prepared in 500 µl DMEM. The internal standards which were used were deuterated testosterone (D2-T; 1.5 ng) and deuterated progesterone (D9-P4; 1.5 ng) (Cambridge Isotope Labs; Cat no. DLM 683 - 0.1 g and Cat no. DLM 7953 - 0.01 g respectively). To each sample, including the standards, a 100 µl of internal standard mix was added. Methyl tert-butyl ether (MTBE) (Sigma; #34875) was added to the sample in a 1:3 ratio, for this experiment that equates to 1.5 ml of MTBE per sample. Samples were vortexed for five minutes and placed in -80°C for 40 minutes, this resulted in a liquid and a solid phase. The liquid phase was decanted into clean test tubes (Lasec; #GLAS1T47M13100) and placed under a nitrogen stream at 45°C to dry. Samples were re-suspended in 300 µl of 50% Methanol (MeOH) (Merck; #1.06009.2500). After which 150 µl of the sample was transferred to mass spectrophotometry (MS) vials (Stargate Scientific; vial #AV5001-0, cap #3.005140.1100.1, insert #3.004025.060E.1) and stored at -20°C until UPLC-MS/MS analysis. For detailed method refer to addendum D.

3.1.5 UPLC-MS.MS conversion

As previously described (Storbeck *et al.*, 2013; Swart *et al.*, 2013) steroids were detected with UPLC (ACQUITY UPLC, Waters, Milford USA) using a Phenomenex Kinetex PFP (2.1 mm x 100 mm x 2.6 mm) column. The mobile phase was made of solvent A and solvent B consisting of 1% formic acid and 49%: 49%:2%, methanol:acetonitrile: isopropanol, respectively (Scholms *et al.*, 2012). The gradients for separations were as indicated by Swart *et al.*, (2013). The total run time was 5 min and the injection volume was 5 µl. For the quantification of steroids the Xevo triple quadrupole mass spectrometer was used. Using an electrospray probe in the positive ionisation mode (ESI+) steroids were assessed with the multiple reaction monitoring (MRM) mode table 3.2. The settings used were as follows: capillary voltage of 3.5 kV, source temperature 120°C, desolvation temperature 400°C, desolvation gas 900 l h⁻¹ and cone gas 50 l h⁻¹. Data was assembled with the Masslynx software version 4.1. A standard curve was produced using the standards and this was used to quantify the concentration of steroid present in each sample.

Table 3.2. UPLC-MS/MS parameters for the separation of four steroid metabolites. Retention time (RT), parent ion, cone voltage (CV), daughter ions and collision energy (CE) is shown.^a Mass transition of the daughter ion used as quantifier; ^b Mass transition of the daughter ion as qualifier.

Steroid metabolite	Retention time (min)	Parent Ion (M+H) ⁺	CV	Daughter Ion ^a (M+H) ⁺	CE	Daughter ion ^b (M+H) ⁺	CE
A4	3.24	287.2	30	96.9	15	108.8	15
T	3.07	289.2	30	97.2	22	109.0	22
D9-P4	4.22	324.2	30	100	20	113	25
D2-T	3.12	291	30	99.1	20	111.25	20

Androstenedione (A4); Testosterone (T); deuterated progesterone (D9-P4); deuterated testosterone (D2-T).

3.2 Experiment 2: Primary Testicular Cell Isolation

As Leydig cells are the primary site for the production of androgens, the aim was to isolate these cells. Testes were obtained from routinely euthenised mice (C657BL/6 mice), collection occurred once they were culled and therefore ethical approval was not required. Testes were kindly provided by the Tygerberg Hospital Animal Unit.

3.2.1 Isolation of Leydig Cells

Leydig cell isolation was performed as previously described by Raucci *et al.*, (2014). Briefly, the testes were decapsulated and incubated in DMEM/F12 (Thermofischer; #BE04-687F/U1), containing 0.25mg/ml collagenase-dispase (Sigma; #10269638001), in a shaking incubator (mrc orbital shaking incubator) for 20 minutes to allow for digestion of connective tissue. This mixture was then removed from the shaking incubator and allowed to settle for five minutes. The contents was then passed through a 70 µm cell strainer (Falcon; #352360) and centrifuged for eight minutes at 250 xg (Eppendorf Centrifuge 5804 R; #5805AK162114). The pellet was washed once with Hanks Balanced Salt Solution (HBSS) (Thermofischer; #14185-048) containing

1mg/ml bovine serum albumin (BSA). The crude suspension was loaded onto a discontinuous Percoll (Sigma; # 10378016) gradient (5ml of 20%; 10ml of 40%, 10ml of 60% and 3ml of 90%; made up in HBSS) before centrifugation for 20 minutes at 800 xg. After centrifugation the third layer from the top was removed and diluted with two parts HBSS-BSA, this was centrifuged for 10 minutes at 350 xg. The pellet was re-suspended in DMEM/F12 (supplemented with 10% FBS and 1% PenStrep).

This method was attempted but did not yield appropriate cell counts for experimental purposes. For this reason as well as support offered by literature (Perrard-Sapori *et al.*, 1987; Lejeune *et al.*, 1998) mixed testicular cultures were performed.

3.2.2 Mixed Testicular Culture

The method was adapted from Hseuh (1980). Briefly, the testes were harvested and each testes pair was placed in an eppendorf containing supplemented DMEM/F12 (1% PenStrep) media. The testes were then decapsulated. This cells mass was placed in a DMEM mixture containing 0.4% Collagenase/Dispase and 0.1% BSA and incubated at 37°C for 90 minutes. The mixture was washed twice with DMEM (10% FBS + 1% PenStrep). The cell mass was evaluated under a microscope and a mixture of cells were observed. After this was established the testis mixture was cultured in supplemented DMEM (10% FBS + 1% PenStrep).

After plating, the cells were allowed to attach for 24 hours. Following this period media was removed and the treatment was initiated. As previously stated the doses of *S. tortosum* used were 0.5mg/ml and 0.01mg/ml. Previous studies by our group noted that 10 μ M Forskolin was capable of stimulating the cells (Smith and Swart, 2016). Therefore this study also stimulated with 10 μ M Forskolin, dissolved in DMSO, (Sigma-Aldrich, #F3917) Pregnenolone (1 μ M, dissolved in DMSO)(Sigma-Aldrich, 5-PREGNEN-3B-OL-20-ONE; #P9129) was administered at to all wells to ensure sufficient reaction substrate. This dosage of PREG was chosen in accordance with other studies which also administered steroids during treatment (Peretz and Flaws, 2013). 10 μ M Citalopram hydroxybromide (known SSRI) was added (Sigma-Aldrich, #C7861) to standard wells only to validate the model and for comparison to potential effects by Trimesemine™. This concentration was decided on, as it has been observed to have effects in studies within our group prior to this one (Coetzee *et al.*, 2016). The treatment design is presented in Table 3.3. After 24 hours the media was collected in glass vials

(Lasec; #G3TUB029Z-013100) and stored at 4°C for steroid extractions. For detailed method refer to addendum B.

Table 3.3. Experimental design for the mixed testicular culture

PREG	Forskolin	Citalopram	<i>S.tortuosum</i>
-	-	-	-
+	-	-	-
+	+	-	-
+	-	+	-
+	-	-	+
+	-	-	+
+	+	-	+
+	+	-	+

Tri (Trimesemine™; mesembrine-rich *S.tortuosum*); Pregnenolone (PREG; 1 µM); Forskolin (10 µM); Citalopram (10 µM).

3.2.3 LC-MS/MS analysis of steroid metabolites

A steroid mix containing A4 and T was used to construct a standard steroid range (0, 0.01, 0.05, 0.01, 0.25, 0.5, 1, 5, 10, 25, 50 and 100 ng/ml) which was prepared in 500 µl DMEM/F12. D2-T (1.5 ng) and D9-P4 (1.5 ng) were the internal standards for this experiment (Cambridge Isotope Labs; Cat no. DLM 683 - 0.1 g and Cat no. DLM 7953 – 0.01 g respectively). Internal standard was added to each sample (100 µl/ sample, including the standards). MTBE (Sigma; #34875) was added to the sample in a 1:3 ratio, therefore 1.5 ml of MTBE was added. Samples were vortexed for five minutes and placed in -80°C for 40 minutes. The liquid phase was decanted into clean test tubes (Lasec; #GLAS1T47M13100) and placed under a nitrogen stream at 45°C to dry. Samples were re-suspended in 300 µl of 50% Methanol (MeOH) (Merck; #1.06009.2500). From this 150 µl of the sample was transferred to mass spectrophotometry (MS) vials (Stargate Scientific; vial #AV5001-0, cap #3.005140.1100.1, insert #3.004025.060E.1) and stored at -20°C until LC/MS analysis. For detailed method refer to addendum D.

3.2.4 UPLC-MS.MS conversion

As previously described (Storbeck *et al.*, 2013; Swart *et al.*, 2013) steroids were detected with UPLC (ACQUITY UPLC, Waters, Milford USA) using a Phenomenex Kinetex PFP (2.1 mm x 100 mm x 2.6 mm) column. The mobile phase was made of

solvent A and solvent B consisting of 1% formic acid and 49%: 49%:2%, methanol: acetonitrile: isopropanol, respectively (Scholms *et al.*, 2012). The gradients for separations were as indicated by Swart *et al.*, (2013). The total run time was 5 min and the injection volume was 5 μ l. For the quantification of steroids the Xevo triple quadrupole mass spectrometer was used. Using an electrospray probe in the positive ionisation mode (ESI+) steroids were assessed with the multiple reaction monitoring (MRM) mode table 3.4. The settings used were as follows: capillary voltage of 3.5 kV, source temperature 120°C, desolvation temperature 400°C, desolvation gas 900 l h⁻¹ and cone gas 50 l h⁻¹. Data was assembled with the Masslynx software version 4.1. A standard curve was generated using the standards and then this curve was used to determine the concentration of steroids within each sample.

Table 3.4. UPLC-MS/MS parameters for the separation of four steroid metabolites. Retention time (RT), parent ion, cone voltage (CV), daughter ions and collision energy (CE) is shown.^a Mass transition of the daughter ion used as quantifier; ^b Mass transition of the daughter ion as qualifier.

Steroid metabolite	Retention time (min)	Parent Ion (M+H) ⁺	CV	Daughter Ion ^a (M+H) ⁺	CE	Daughter ion ^b (M+H) ⁺	CE
A4	3.24	287.2	30	96.9	15	108.8	15
T	3.07	289.2	30	97.2	22	109.0	22
D9-P4	4.22	324.2	30	100	20	113	25
D2-T	3.12	291	30	99.1	20	111.25	20

Androstenedione (A4); Testosterone (T); D9-P4 (deuterated progesterone); D2-T (deuterated testosterone).

3.3 Experiment 3: Primary Ovarian Culture

As there is still debate regarding the classification of *S.tortuosum*, some arguing it is a SSRI while other state it is a monoamine releasing agent (MRA), it was necessary to evaluate the effect on estrogens as well. There have been reports that both these drug classes have endocrine disrupting abilities (Barenys *et al.*, 2009).

3.3.1 Ovarian Mixed Culture

Animals were kindly donated by the Tygerburg Hospital Animal Unit. The animals were routinely euthenised, and access was granted once they were euthenised. For this study the mice used were wild type C57/BL/6 female mice. It was not established during what phase of their cycle they were prior to culling, from our raw data however this did not appear to skew the results as all of the control had the same E2 levels.

For the production of estrogens both the theca and the granulosa cells are required, owing to this a mixed ovarian culture was decided on as a model.

The method was adapted from Magoffin and Erickson (1988). Briefly, ovaries were isolated and collected in supplemented DMEM/F12 (1% PenStrep) media, each ovary pair was collect in separate eppendorf tubes. The ovaries were washed with DMEM/F12, to prevent contamination and then cut into pieces using sterilised scissors. The minced ovaries were incubated in supplemented DMEM/F12 (1% PenStrep + 1mg/ml BSA + 0.02% Collagenase/Dispase) for two hours, this mixed was disturbed with a Pasteur pipette every 30 minutes. After the two hours the cells were passed through a cell strainer (70 μ m) and centrifuged at 250 xg for five minutes. The pellet was washed twice with DMEM/F12.

The cells were plated at a concentration of 4×10^5 cells per well with supplemented DMEM/F12 media (10% FBS + 1% PenStrep), they were incubated at 95% CO₂ and 5% N₂, at 37°C. The cells were allowed to attach for 24 hours. Following this period the treatment protocol was started. Cells were treated under the following conditions: 10 μ M Citalopram hydroxybromide, 10 μ M Forskolin, high dose *S.tortuosum* (0.5 mg/ml) and low dose *S.tortuosum* (0.01 mg/ml), with the addition of PREG (1 μ M) for certain conditions, as depicted in table 3.5. After 24 hours of treatment, 500 μ l of supernatant was collected in glass vials at stored at 4 °C, prior to steroid extractions. For detailed method refer to addendum C.

Table 3.5. Experimental layout for ovarian mixed culture

	Citalopram	Forskolin	Pregneneolone	<i>S.tortuosum</i>
Control	-	-	-	-
Forskolin	-	+	-	-
Citalopram	+	-	-	-
Tri high dose	-	-	-	+
Tri low dose	-	-	-	+

Tri high dose + Fors	-	+	-	+
Tri low dose + Fors	-	+	-	+
Tri high dose + PREG	-	-	+	+
Tri low dose + PREG	-	-	+	+

Tri (Trimesemine™; mesembrine-rich *S. tortuosum*); Fors (Forskolin; 10 µM); PREG (Pregnenolone; 1 µM); Citalopram (10 µM).

3.3.2 LC-MS/MS analysis of steroid metabolites

A steroid mix containing Estrone (E1) and Estradiol (E2) was used to construct a standard steroid range (0, 0.01, 0.05, 0.01, 0.25, 0.5, 1, 5, 10, 25, 50 and 100 ng/ml) which was prepared in 500 µl DMEM/F12 media. The internal standards which were used were D2-T (1.5 ng) and D9-P4 (1.5 ng) (Cambridge Isotope Labs; Cat no. DLM 683 - 0.1 g and Cat no. DLM 7953 - 0.01 g respectively). Each sample, including the standards, received 100 µl of internal standard mix. MTBE (Sigma; #34875) was added to the sample in a 1:3 ratio, therefore 1.5 ml of MTBE was added per sample. Samples were vortexed for five minutes and placed in -40°C for an hour, to ensure that the solid and liquid phase separate. The liquid phase was decanted into clean test tubes (Lasec; #GLAS1T47M13100) and placed under a nitrogen stream at 45°C to dry. Derivatisation of samples have been reported to enhance the detection of the estrogens (Nelson *et al.*, 2004). For the purpose of this study samples were derivatised with dansyl chloride using a method previously performed in laboratory of Prof AC Swart. Samples were re-suspended in 100 µl Na₂CO₃ (0.1 M). 100 µl of a dansyl chloride solution (2 mg/ml) was added to each sample. The samples were vortexed and placed in a pre-heated oven at 60 °C for two minutes. Once removed from the oven, 150 µl of the sample was then transferred to mass spectrophotometry (MS) vials (Stargate Scientific; vial #AV5001-0, cap #3.005140.1100.1, insert #3.004025.060E.1) and stored at -20°C until LC/MS analysis. For detailed method refer to addendum E.

3.3.3 UPLC-MS.MS conversion

As previously described (Storbeck *et al*, 2013; Swart *et al*, 2013) steroids were detected with UPLC (ACQUITY UPLC, Waters, Milford USA) using a Phenomenex Kinetex PFP (2.1 mm x 50 mm) column. The mobile phase was made of solvent A and solvent B consisting of 1% formic acid and 49%: 49%:2%, methanol: acetonitrile: isopropanol, respectively (Scholms *et al.*, 2012). The gradients for separations were as indicated by Swart *et al* (2013).The total run time was 4.2 min and the injection volume was 5 µl. For

the quantification of steroids the Xevo triple quadrupole mass spectrometer was used. Using an electrospray probe in the positive ionisation mode (ESI+) steroids were assessed with the multiple reaction monitoring (MRM) mode table 3.6. The settings used were as follows: capillary voltage of 3.5 kV, source temperature 120°C, desolvation temperature 400°C, desolvation gas 900 l h⁻¹ and cone gas 50 l h⁻¹. Data was assembled with the Masslynx software version 4.1. Standard curves were produced for each steroid metabolite with the following concentrations: 0, 0.01, 0.05, 0.1, 0.25, 0.5, 1, 5, 10, 25, 50, 100 ng/ml. The method was adjusted from one which was developed by Dr. Storbeck's (Department of Biochemistry, Stellenbosch University) group in 2016 (UPLC2016BEHDansyl).

Table 3.6. UPLC-MS/MS parameters for the separation of four steroid metabolites. Retention time (RT), parent ion, cone voltage (CV), daughter ions and collision energy (CE) is shown.^a Mass transition of the daughter ion used as quantifier; ^b Mass transition of the daughter ion as qualifier.

Steroid metabolite	Retention time (min)	Parent Ion (M+H) ⁺	CV	Daughter Ion ^a (M+H) ⁺	CE	Daughter ion ^b (M+H) ⁺	CE
E1 (estrone)	3.2	504	20	156	45	171	35
E2 (β-Estradiol)	3.0	506	35	156.00	40	171.00	35
D9-P4	4.22	324.2	30	100	20	113	25
D2-T	3.12	291	30	99.1	20	111.25	20

Estrone (E1); Estradiol (E2); Deuterated Progesterone (D9-P4); deuterated testosterone (D2-T).

3.4 Statistical Analysis

Statistical analysis was performed using Microsoft Excel 2010 and STATISTICA 64 (TIBCO, USA). After basic statistics confirmed normality a One-way or Factorial ANOVA, as well as student t-tests were employed, as applicable. Where ANOVA returned significant main effects, specific differences between experimental conditions were compared using Fisher LSD post hoc tests. Significance was set at p<0.05. Each experiment had three technical repeats and each repeat was conducted in triplicate. All data was reported using standard error of the mean (SEM).

CHAPTER 4

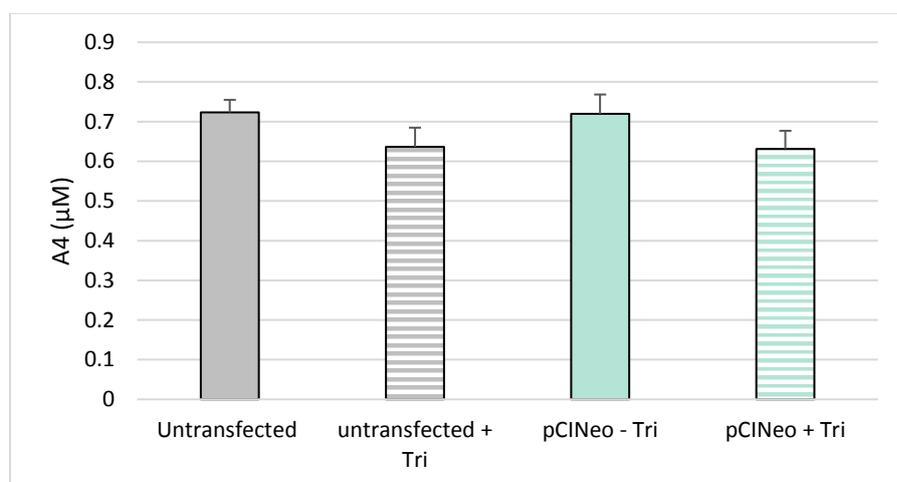
RESULTS

4.1 Experiment 1: Transfected HEK293 cultures

4.1.1 Effect on Androstenedione (A4)

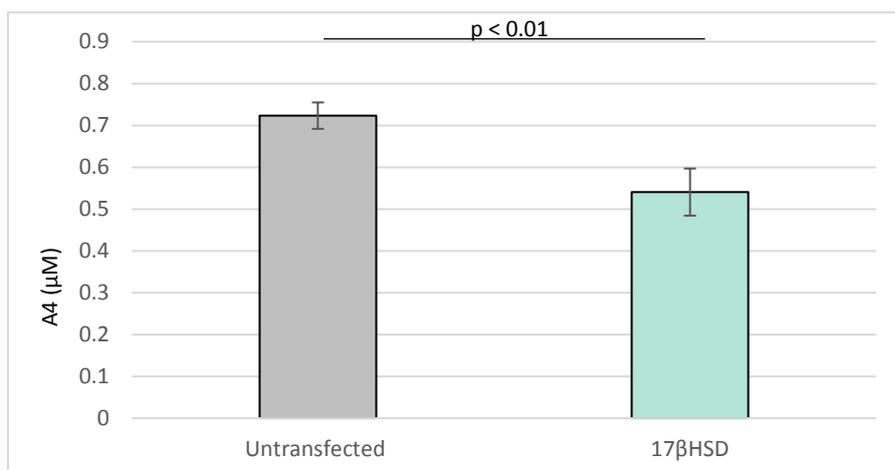
Human embryonic kidney (HEK293) cells were used for the transfections. This model was specifically chosen as Prof AC Swart's laboratory has performed numerous successful transfections using these cells. Additionally, it also allowed the specific enzyme under investigation to be over-expressed, thereby ensuring the detection of any deviations from its normal function. To determine whether there is an effect of the transfection vehicle (pCINeo), the untransfected cells were compared to cells which were transfected with pCINeo, in the presence and absence of Tri™ (0.5 mg/ml). pCINeo showed no effect on A4 production (Fig.4.1).

Fig.4.1. Confirmation that the vehicle used did not affect A4 production or the effect of Trimesemine™.



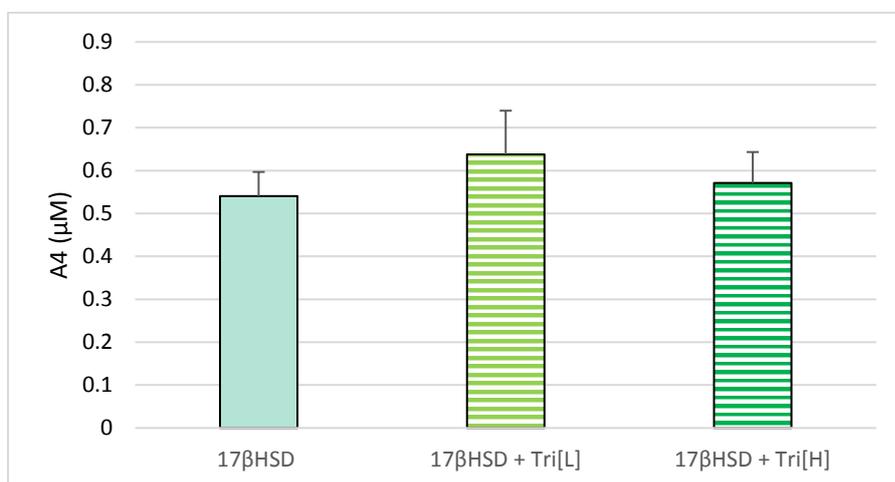
No significant difference between the untransfected and the vector transfected groups in the presence and absence of Tri™. The verticle lines represent the groups treated with Tri™ (0.5 mg/ml)

The 17βHSD₅ enzyme is responsible for the conversion of A4 to T. Thus, for this model to be validated the concentration of A4 was assessed, as a successful transfection would result in a decreased concentration of A4. From the results in Fig.4.2. it can be seen that the concentration of A4 was significantly ($p = 0.0066$) reduced after transfection. These results not only validate the model but also indicate that the transfection was a success.

Fig.4.2. Effect of transfection with 17 β HSD on production of A4.

A significant decrease was noted after transfection with the plasmid 17 β HSD, as expected. This validated the model.

In order to establish whether Trimesemine™ has an effect on the concentration of A4, an one-way ANOVA was performed on the 17 β HSD₅ transfected cells. None of the doses assessed had any significant effect on the concentration of A4 in the transfected cells (Fig.4.3).

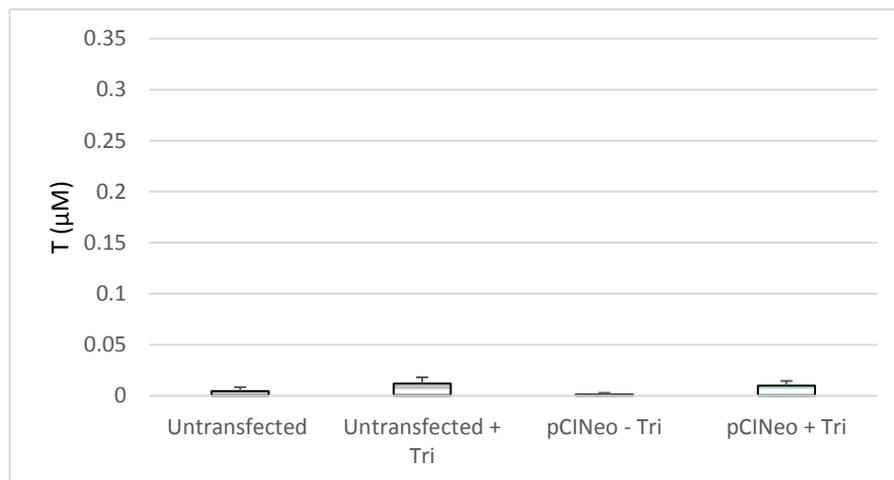
Fig.4.3. Effect of Trimesemine™ on the concentration of A4, in 17 β HSD₅ transfected cells.

Trimesemine™ (Tri); Tri(L) (Trimesemine™ low dose; 0.01 mg/ml); Tri(H) (Trimesemine™ high dose; 0.5 mg/ml). No significant difference was present ($p = 0.715$).

4.1.2 Effect on testosterone (T)

Similar to A4 data, there was no significant difference between the untransfected cells and those transfected with the vehicle control (Fig.4.4) regarding the concentration of T. Furthermore, TriTM did not affect T levels at any dose tested in untransfected and pCINeo transfected cells. T production was very low under all conditions, as expected.

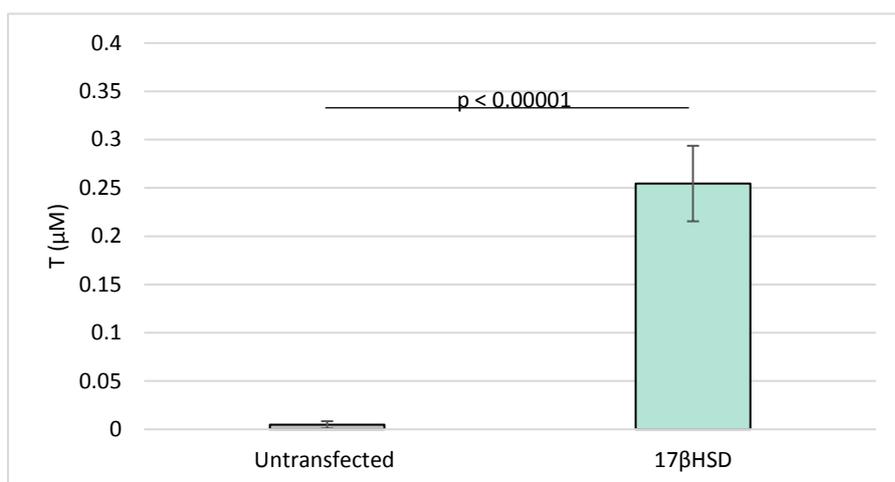
Fig.4.4. Confirmation that the vehicle used did not affect T production or the effect of TrimesemineTM



TriTM (TrimesemineTM). No significant difference in T concentration in the presence of ST in untransfected ($p = 0.21$) and pCINeo vector ($p = 0.10$). There was also no effect ($p = 0.24$) of the vehicle (pCINeo) control on the concentration of T. Vertical lines represent the addition of TriTM (0.5 mg/ml).

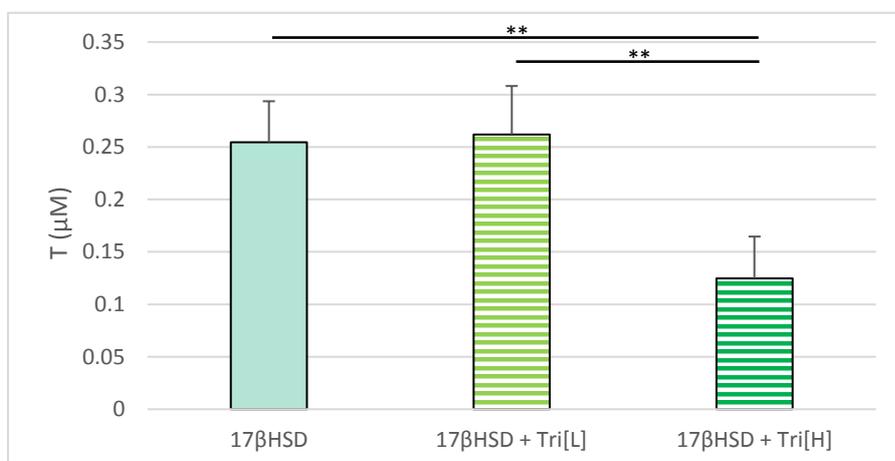
The next step was to validate the model by determining whether the transfection produced an increase in the concentration of T. Fig.4.5. indicates a significant increase in the T concentration from basal (untransfected) levels to those cells in which the converting enzyme is over expressed.

Fig.4.5. Effect of enzymatic transfection on T production.



Significant increase in the T concentration after transfection, $p = 0.0000101$.

To determine if TriTM had an effect on the concentration of T the transfected cells were treated with low (0.01 mg/ml) and high (0.5 mg/ml) doses of TriTM. Fig.4.6 shows that there is a significant decrease in T at high doses compared to control and low doses.

Fig.4.6. Effect of TriTM on T production.

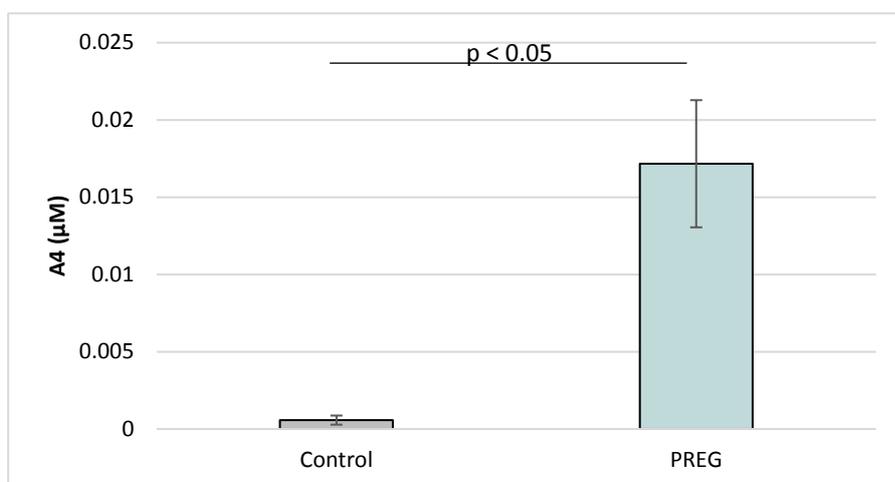
Tri (TrimesemineTM a mesembrine-rich *S.tortuosum*). Tri[L] (TrimesemineTM low dose, 0.01 mg/ml). Tri[H] (TrimesemineTM high dose, 0.5 mg/ml). # represents a significant difference ($p < 0.05$; $p = 0.018$) between the 17βHSD₅ in the absence of Tri compared to high dose Tri. While the ** indicates the significant difference ($p < 0.05$; $p = 0.021$) present between the low and high doses of TriTM.

4.2 Experiment 2: Primary testis mixed cell culture

4.2.1 Effect on A4 synthesis

Primary cultures were used to determine whether these effects remained prevalent in the tissues responsible for the primary production of gonadocorticoids. Cells were stimulated with PREG to determine if an increase in the basal steroid production occurred. In Fig.4.7 it is evident that there is a significant increase in the concentration of A4 compared to control.

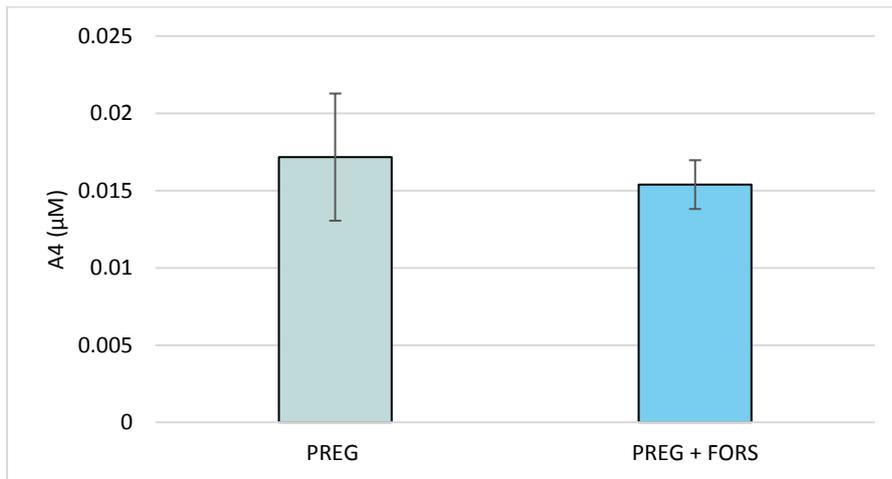
Fig.4.7. Effect of pregnenolone on A4 production in testicular cultures



There was a significant increase ($p = 0.012$) between control compared to PREG (Pregnenolone stimulated) groups.

Another agent known to upregulate adrenal steroidogenesis is Forskolin. As this is known to upregulate the biosynthetic machinery, PREG was added together with Forskolin with this treatment to prevent possible substrate depletion. To determine whether there was increased androgen production in testicular tissue after Forskolin stimulation, a student t test was carried out. Forskolin had no significant effect on the production of androgens, as noted in Fig.4.8. This together with the results reported above, resulted in PREG being the stimulant of choice in all subsequent treatments.

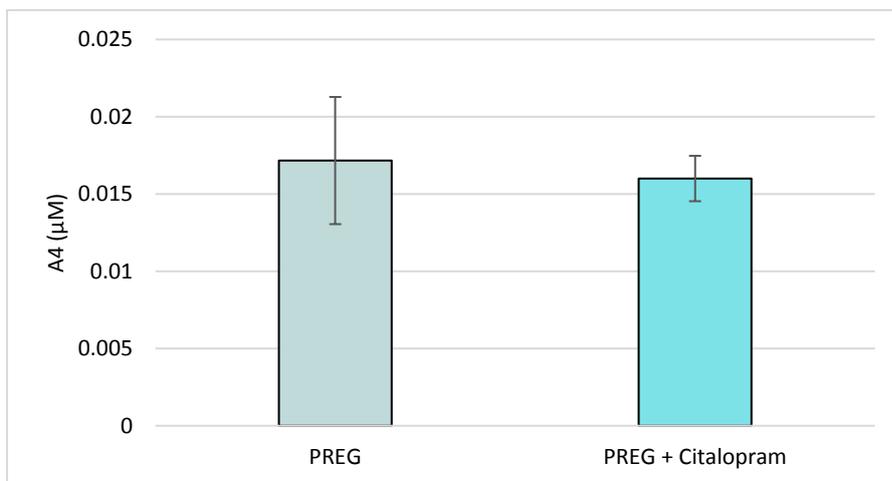
Fig.4.8. Effect of Forskolin on A4 steroid synthesis in the testicular tissue.



$p = 0.33$, no significant effect with the use of Forskolin (FORS) + PREG compared to PREG alone.

There was no significant effect on the concentration of A4 in the presence of Citalopram (Fig.4.9).

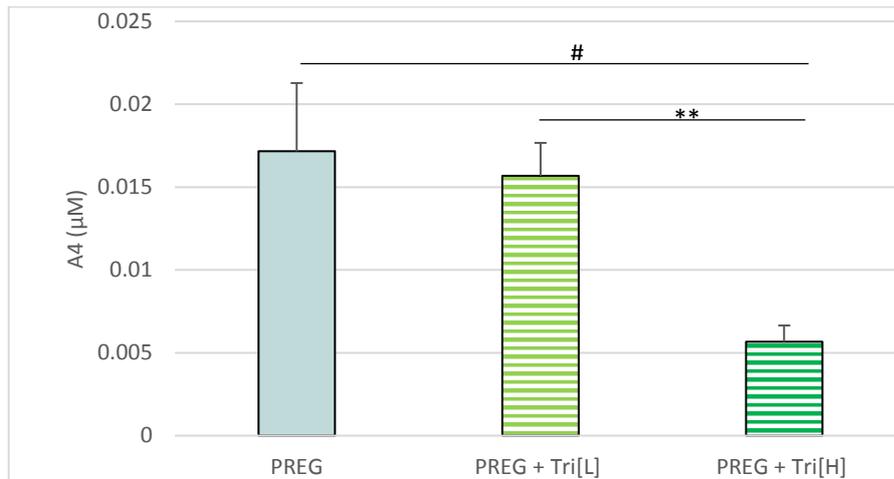
Fig.4.9. Effect of citalopram on the concentration of A4 in testicular cultures.



$p = 0.76$; there is no significant difference in PREG + Citalopram (10 µM) compared to PREG alone.

Trimesemine™ at low doses did not have an effect relative to the control (PREG group), however at high doses there was a significant difference in the concentration of A4, in comparison to the control as well as the low dose group (Fig.4.10).

Fig.4.10. Effect of Trimesemine™ on A4 production in testicular cultures.

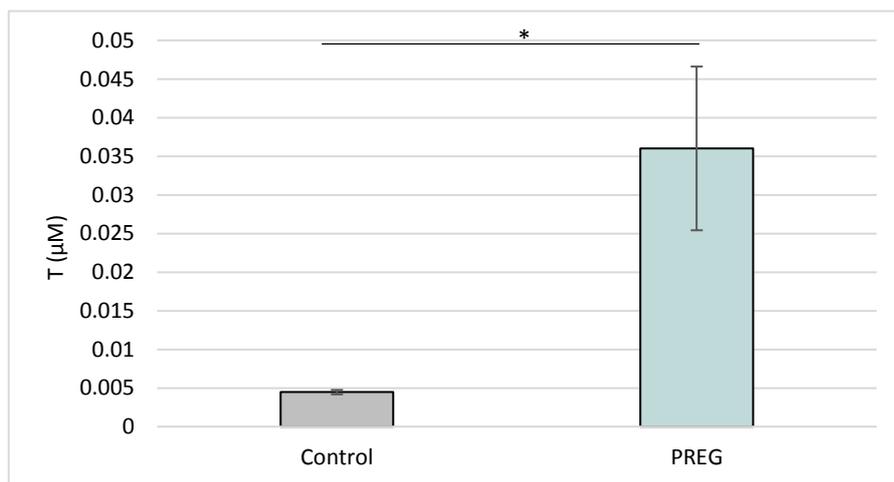


$p = 0.74$, therefore no significant difference with the low dose Tri (Trimesemine™, 0.01 mg/ml). Significant difference ($\# = p < 0.05$; $p = 0.04$) with the high dose Tri (Trimesemine™, 0.5 mg/ml) compared to control (PREG) and low dose Tri ($** = p < 0.05$; $p = 0.011$).

4.2.2 Effect on testosterone synthesis

To determine whether the increased androgen synthesis was sustained in the presence of PREG, a student t-test was used to assess this. From Fig.4.11. it can be observed that there is a significant increase in the concentration of T synthesis.

Fig.4.11. Effect of PREG on T production in testicular cultures



* = $p < 0.05$. Significant increase ($p = 0.014$) with PREG stimulation.

In terms of T production, in the presence of PREG, similar results were obtained in the presence of Forskolin and Citalopram, with neither condition affecting T synthesis (Fig.4.12. and Fig.4.13. respectively).

Fig.4.12. Effect of Forskolin on the concentration of T in the presence of PREG in testicular cultures.

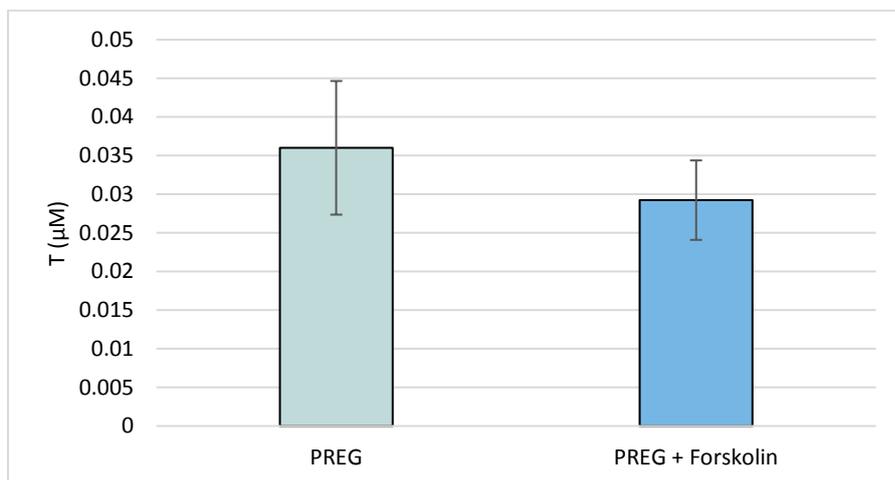
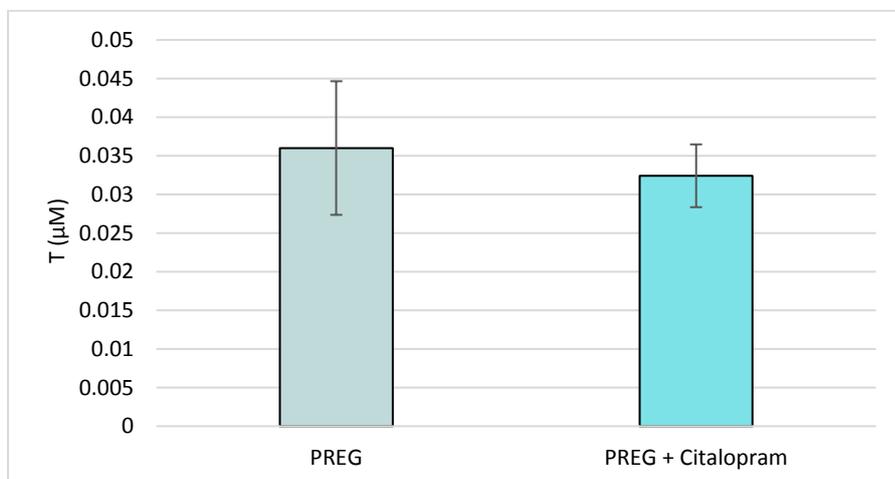
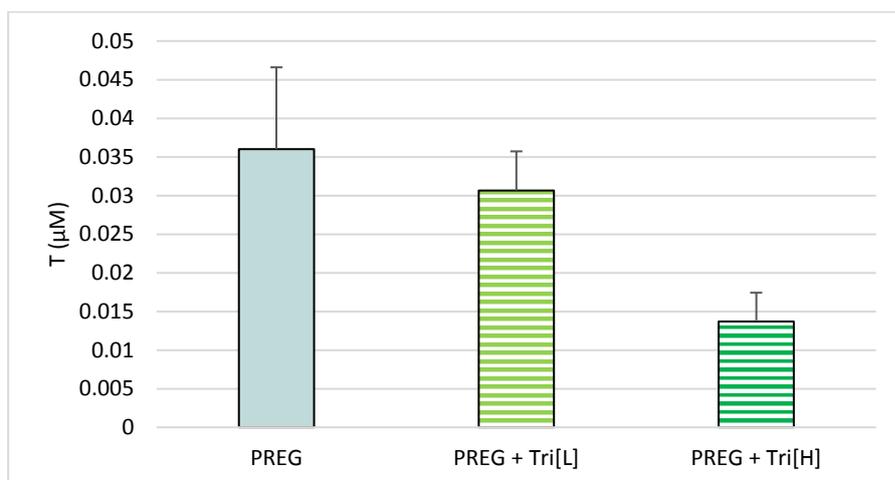


Fig.4.13. Effect of Citalopram on the concentration of T in the presence of PREG in testicular cultures.



Trimesemine™ did not appear to have a significant effect on the concentration of T in the under PREG stimulation (Fig. 4.14).

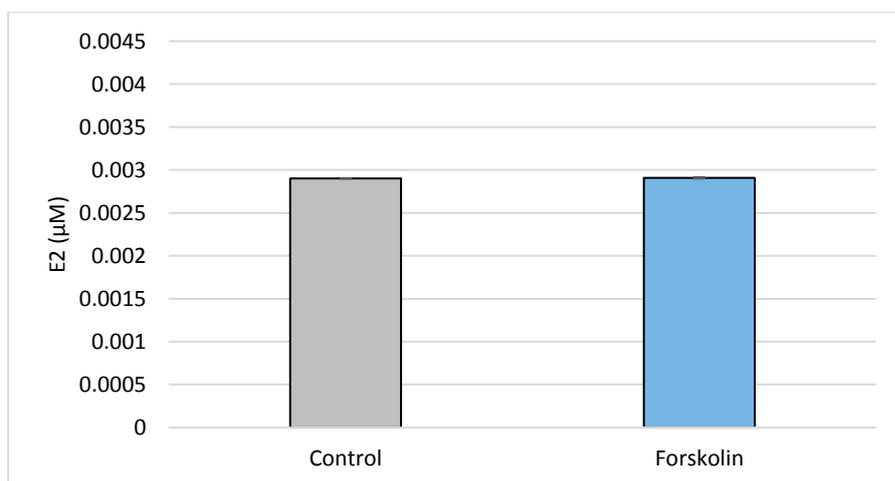
Fig.4.14. Effect of Trimesemine™ on T production in testicular cultures.



4.3 Experiment 3: Primary ovarian mixed cell culture

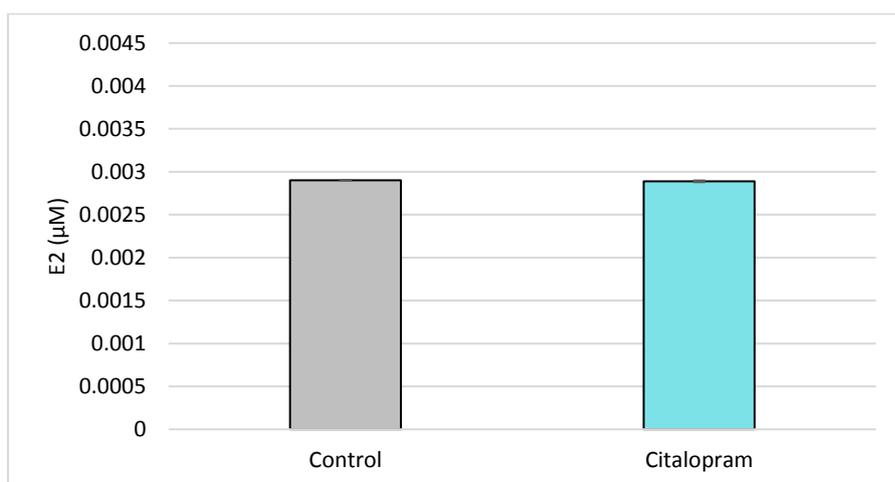
The ovarian crude culture was treated with Forskolin in an attempt to upregulate steroidogenesis, but – similar to testis data – Forskolin did not appear to have an effect on estrogen (specifically E2) biosynthesis (Fig.4.15). In these experiments PREG is not present unless otherwise stated.

Fig.4.15. E2 production under control vs. Forskolin conditions.



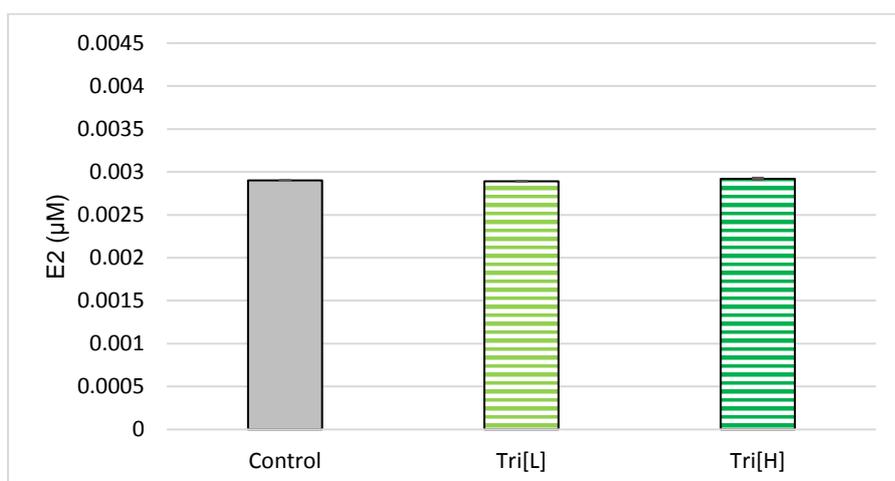
Similar to the results obtained in the testes mixed cultures, citalopram did not appear to have an effect on E2 synthesis at the dosage tested (Fig. 4.16).

Fig.4.16. E2 production under control vs Citalopram conditions in ovarian mixed culture.



An ANOVA was performed to assess the effect of Trimesemine™ on the synthesis of E2 in the ovarian mixed culture. Although there was not significant difference reported relative to the control (Fig.4.17).

Fig.4.17. Effect of Trimesemine™ on E2 synthesis in ovarian mixed culture.



Tri[L] (Trimesemine™, low dose 0.01 mg/ml); Tri[H] (Trimesemine™, low dose 0.5 mg/ml)

To determine whether the presence of PREG could increase ovarian steroidogenesis, PREG was added together with Citalopram and Tri at low and high doses. However the presence of PREG did not affect the concentration of E2, reported in Fig.4.18. and Fig.4.19. respectively.

Fig.4.18. Effect of citalopram on concentration of E2 in ovarian mixed culture.

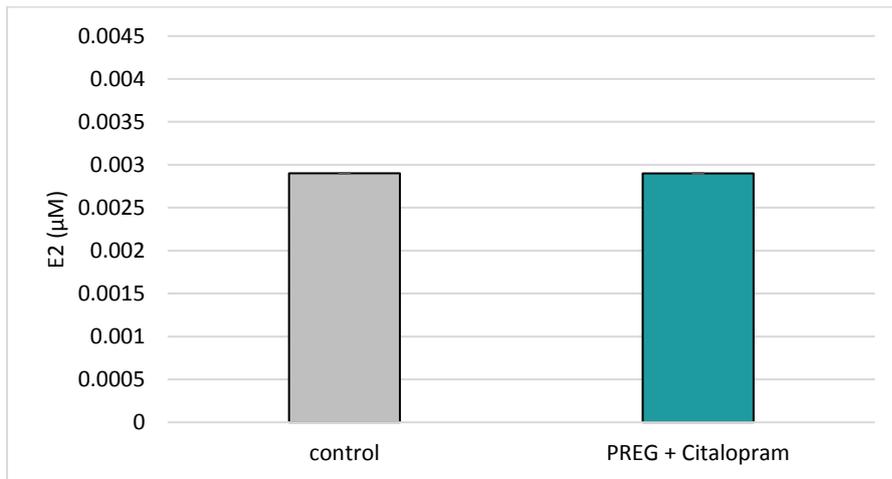
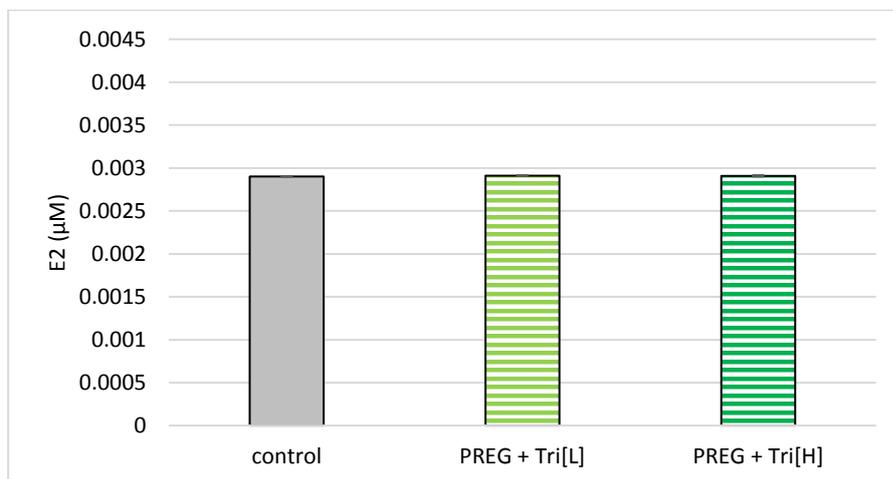


Fig.4.19. Effect of Trimesemine™ (Tri) on E2 production in the presence of PREG in ovarian mixed culture.



Pregnenolone (PREG); Tri[L] (Trimesemine™ low dose, 0.01 mg/ml); Tri[H] (Trimesemine™ high dose, 0.5 mg/ml).

Although an attempt was made to also assess estrone (E1) levels, levels for this hormone was not within the detectable range.

CHAPTER 5

DISCUSSION

In the context of anxiety and depression, many positive effects of *S.tortuosum* extracts have been reported, by more than one research group (Terburg *et al.*, 2013; Loria *et al.*, 2014; Harvey *et al.*, 2011; Smith, 2011). A recent study by our group suggested that the extract may have potential negative effects on androgen production (Smith and Swart, 2016), although this result was not obtained in H295R cells and not in gonadal tissue. Nevertheless, since antidepressant and anxiolytic drugs often have endocrine disruptive effects (Mennigen *et al.*, 2008; Ilgin *et al.*, 2017; Müller *et al.*, 2012; Safarinejad, 2008), it was imperative to elucidate this potential side-effect of *S.tortuosum*.

In the first experiment, potential effects of a mesembrine-rich *S.tortuosum* (Tri™) on 17βHSD₅ were investigated. Human embryonic kidney (HEK293) cells were used as these have proved effective with transient expression of enzymes involved in the biosynthesis of steroids (Panter *et al.*, 2005; Toit, 2015; Barnard, 2017). To exclude potential confounding effects from the transfection protocol itself, we confirmed that the vector (pCINeo) alone did not have an effect on the steroid synthesis and the quantity of the measured steroids A4 and T were similar to that of the untransfected cells. Therefore, the changes which were observed can be attributed to the overexpressed enzyme (17βHSD₅).

17βHSD₅ is responsible for the conversion of A4 to T, but also assists the reverse reaction from T to A4. To validate the model, it was necessary to determine whether the overexpression of 17βHSD₅ resulted in detectable changes in the steroid concentrations produced in culture. This was indeed the case, as there was more A4 present in the untransfected cultured HEK293 cells compared to the 17βHSD₅ transfected cells, with the opposite result for T, indicating that this transfection favours the conversion of A4 to T.

After validating the model, the effects of Tri™ was assessed. Tri™ was administered at a proven effective dose (0.01 mg/ml) and a high dose (0.5 mg/ml). These doses are within a narrower range than doses used previously (Smith and Swart, 2016; Coetzee *et al.*, 2016), but the 0.01 mg/ml dose – which was also included as calculated from the recommended dose suggested by manufacturers – is the dose which has

consistently been shown to have beneficial effects. The higher dose was included here to enable illustration of potential dose-dependent effects. Current results suggest that the recommended dose did not have disruptive effects in the context of A4 and T. The high dose of Tri™ appeared to inhibit conversion of A4 to T, suggesting inhibition of the 17 β HSD₅ enzyme. To conclusively establish an inhibitory effect of 17 β HSD₅ it is necessary to evaluate the concentration of DHEA relative to androstenediol, as this enzyme is required for this conversion as well. It is noteworthy that the decrease in T observed with the high dose is not as a result of substrate depletion – the A4 data at high dose Tri™ is not different from the control. As the general concentration of A4 was greater than that of T, it could be possible that undetectable changes in A4 synthesis can transmute to observable changes in the concentration of T. This interpretation is in line with previous reports by Smith and Swart (2016), who reported a dose of 0.0001 mg/ml to decrease the production level of A4 significantly in adrenal cells. It is important to note however that these cells (H295R) were not similar to the cells used in this study (HEK293), so that treatment doses may not necessarily be directly comparable, as H295R cells could be more sensitive, given their inherent ability for *de novo* steroid synthesis (Scholms *et al.*, 2012, (Nielsen *et al.*, 2015). This model supports the notion that mesembrine-rich *S.tortuosum* at high doses may have the ability to inhibit the 17 β HSD₅ enzyme.

H295R and HEK293 cell lines are commonly used to investigate enzyme inhibition activity, however neither of these cell models are analogues to the primary site of gonadocorticoid production. Thus, in an attempt to elucidate the effects of *S. tortuosum* in more relevant models, primary gonadal mixed cell cultures were used to further elucidate androgenic or estrogenic effects in the tissues responsible for their primary production (testes and ovaries, respectively).

Initially, Leydig cell isolation were attempted as the model for male gonadal culture. However, this proved more difficult than expected and yielded a low cell viability. This was possibly unsuccessful owing to the small size of the testes, as most studies performing primary isolations used larger experimental animals, such as rats and pigs (Seaz *et al.*, 1983; Raucci *et al.*, 2014). Crude cultures of testicular tissue have been carried out and yielded results similar to those of crude cultures, for these reasons mixed cell cultures were chosen (Hsueh, 1980; Saez, 1983). The mixed culture produced enhanced cell viability. At the point during which the experiments were carried out Leydig cells were not in our possession, since then Leydig cells have been

acquired, and future studies will make use of them. These cells were kindly donated by Prof R. Henkel (university of the Western Cape). Addition of PREG to culture conditions increased steroid synthesis significantly for both the production of A4 and T as also reported in the literature (Hall *et al.*, 1987). In contrast, although the addition of Forskolin is known to generally stimulate steroid production in H295R cells (Nelson *et al.*, 1999; Chaturvedi *et al.*, 2004; Scholms *et al.*, 2012; Smith and Swart, 2016), it did not have a significant effect on gonadal cell culture. Therefore, PREG was used to stimulate the steroid pathway instead of Forskolin.

Citalopram was chosen as a standard SSRI treatment, as a comparison to *S.tortuosum*. Literature has reported that drugs with SSRI effect possess endocrine disrupting effects, which is most often reflected as a decrease in T levels (Gregorian *et al.*, 2002; Safarinejad, 2008; Erdemir *et al.*, 2014; Taylor and Balon, 2014;). According to current results, there does not appear to be a significant difference with the administration of 10 μM Citalopram on the concentration of A4 or T detected from the testicular cells, nor does it appear to alter the E2 biosynthesis of the ovarian cells. We have previously shown this dose of Citalopram to exert SSRI effects *in vitro* (Coetzee *et al.*, 2016). However, it is possible that this dose of citalopram was too low to illustrate effects on T production. This is supported by literature from Hansen *et al.*, (2017) and Müller *et al.*, (2012) who reported significant decreases in T concentration at doses of 10 μM (Citalopram) and significant estrogenic effects at 17 μM .

There is limited information regarding the changes in estrogens with the administration of SSRI on female sex hormones, however some have reported estrogenic effects for flovoxetine (Mennigen, 2008; Müller *et al.*, 2012). Although results from this study did not yield significant changes with the administration of 10 μM Citalopram, but this could simply be due to the concentration being too low. Our data is in accordance with Hansen *et al.*, (2017) who also did not observe changes in E2 at a dose of 10 μM citalopram. Müller *et al.*, (2012), however reported effects at 17 μM using another SSRI, flovoxetine. It has been reported that the serum concentrations for citalopram range from 15-124 ng/ml, thus the dosages which are used may never be achieved in the body (Devane, 1999). Contradictory to this Hodgson *et al.*, (2013) reported that there is not a linear relationship between the therapeutic effect and the serum concentration of the SSRI. Nevertheless, taken together, in retrospect, a higher concentration of citalopram may have produced more suitable results for comparison.

In terms of the medicinal plant under investigation, *S.tortuosum* was shown in both the HEK293 and primary testis culture models, to decrease A4 and/or T concentration at the high dose only, while E2 production by ovarian cells were not significantly affected at either dose. The reduction of A4 (testicular culture) with the high dose of Tri™ is suggestive of an upstream inhibition of the 3 β HSD enzyme, as proposed by Smith and Swart (2016). With the decrease in T (HEK293 cells) at this dose, the inhibition of 17 β HSD₅ cannot be negated.

While no changes were observed for E2, which is a positive finding, as others with similar mechanisms of action (MRA or SSRI) have estrogenic activities. As mentioned earlier E1 was assessed but when it was measured it was below the detectable range. A possible explanation for the poor detection of E1, could suggest there is an increase in the activity of the 17 β HSD, indicative of an estrogenic effect. One would expect an increase in the concentration of E2 relative to the control, yet there was no statistical difference found. Taking the androgen (A4 and T) results into consideration which suggests an inhibition of 17 β HSD, the increased 17 β HSD is unlikely. The poor detection of E1 could simply be explained by the model, which may not have been sensitive enough to allow the detection of E1, another possible explanation could be that E1 occurs predominantly in postmenopausal women. E2 is the more potent of the two steroids and is required for a host of functions.

S.tortuosum has been shown to have both SSRI (Harvey *et al.*, 2011; Terburg *et al.*, 2013) and monoamine releasing effects (Coetzee *et al.*, 2015). It is therefore necessary to put current results into context of literature on both SSRIs and MRAs. From current results, it appears that Tri™ does not have similar effects as those reported for SSRI (as already discussed in the context of the citalopram data), although only at the high dose (0.5mg/ml) which far exceeds the dose recommended for treatment. Therefore, these androgen-related side-effects should probably not be experienced by individuals after *S.tortuosum* consumption. In addition, given the high risk of overdosing in patients suffering from depression, a decreasing A4/T level over time may be a potential indicator of accidental overdosing. This possibility should be further elucidated using long-duration treatment protocols in appropriate rodent models. Similar, in the context of the SSRI literature, E2 data suggest very little, or no risk, for endocrine disruption in female consumers of the extract.

Turning attention now to MRA-related endocrine disruption, several MRAs have been reported to have estrogenic effects. For example, methamphetamines, including modern “party drugs” such as BZP and TFMPP, were reported to increase estrogen regulating elements, reflected as increased proliferative effect in the estrogen-sensitive MCF-7 breast cell line (Corrodi *et al.*, 1967; Min *et al.*, 2012), suggesting an increased sensitivity to estrogen in response to MRA exposure.

Publications could not be found which focused on the effects of MRA drugs on actual estrogen levels, perhaps due to the general lack of research using female subjects, which is only starting to be addressed in the literature. One relatively old study in ovariectomised rodents reported that administration of estradiol benzoate decreased monoamine oxidase activity in the female brain (Luine *et al.*, 2017). Although this suggests cross-talk between the monoamine and androgen systems, subsequent literature seems to have largely neglected to report data which may further contextualise current data. Thus, to err on the side of caution, although current data does not suggest a decrease in estrogen levels to be a potential side-effect of *S.tortuosum*, the possibility of an effect at the level of estrogen receptors, which may modulate estrogen sensitivity, remains to be elucidated.

CHAPTER 6

CONCLUSION

Taken together, current data suggest that gonadal tissue may be less sensitive to the inhibitory effect of *S.tortuosum* previously illustrated in adrenal cell T synthesis. Secondly, the recommended dose of *S.tortuosum* does not appear to change gonadal production of either male or female sex hormones.

Therefore, *S.tortuosum* extract may be superior to other antidepressant drugs in that it does not result in androgenic or estrogenic responses, in doses recommended for the treatment of depression and anxiety.

Although this cell study suggests there is no endocrine disruptive effect in terms of the sex steroid production, in both genders, conclusive evidence requires further *in vitro* studies on gonad specific cell line as well as *in vivo* experiments.

CHAPTER 7

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ADDENDUM A

SOP: Transfection of HEK293 Cells

- Seed 1×10^6 HEK293 cells in T75 cultures flasks
- Media used = DMEM + 1% PenStrep + 10% FBS
- Grow cells until they are 3 passages from the initial passage (eg. P15 – P18)
- Plate the cells at 2.5×10^5 cells per well in a 24-well culture plate
- Leave for 24 hours to attach
- Transfect cells with DNA using the XtremeGene Transfection Reagent
- Transfection reagent to DNA ratio is 3:1
- Final reaction volume per well should be 500 μ l
- Media for transfection must be unsupplemented, for proper transfection
- Leave in this media for 48 hours
- After 48 hours remove the transfection media
- Add the treatment media, making sure each well has a total of 600 μ l media, to account for evaporation
- After 24 hours remove 500 μ l of supernatant and place in glass test tubes
- Store at 4 °C until the point of steroid extraction – don't store for longer than 2 weeks

ADDENDUM B

SOP: Testicular Mixed Culture

- Collect the testes in cold supplemented media DMEM/F12 (1% PenStrep + 0.1% Gentamycin) – each pair to be collected in its own tube
- Decapsulate testes and place the cell mass in DMEM/F12 media containing 0.4% Collagenase/Dispase and 0.1% BSA
- Place in the shaking incubator
- Allow enzyme digestion to take place to 90 minutes, pipetting at 30 intervals
- After 90 min, wash the cells 3 times at 1500 rpm for 5 min in DMEM/F12
- Cells were plated at 5×10^5 cells/ well in supplemented DMEM/F12 (1% PenStrep) in a 48 well plate
- Allow the cells to recover for 24 hours in supplemented DMEM
- Remove the media and add the desired treatment media
- Leave the cells in the treatment media for 24 hours
- Collect supernatant after 24 hours and store in in the glass vials
- Sores the samples at 4 °C until the point of steroid extraction

***Adapted from Hseuh (1980).

ADDENDUM C

SOP: Ovarian Mixed Culture

- Collect the testes in cold supplemented media DMEM/F12 (1% PenStrep + 0.1% Gentamycin) – each pair to be collected in its own tube
- Cut the ovaries into pieces (4 or more)
- Place the ovaries in DMEM/F12 containing 2% Collagenase/Dispase and 1 mg/ml BSA
- Place in the shaking incubator
- Leave for 2 hours, pipetting at 30 min intervals
- Pass the ovaries through a cell strainer
- After the incubation wash 3 times with DMEM/F12 at 250 g for 5min
- Plate at Cells are plated at 5×10^5 cells/ well in supplemented DMEM/F12 (1% PenStrep + 10% FBS) in a 48 well plate
- Allow the cells to recover for 24 hours in supplemented DMEM
- Remove the media and add the desired treatment media
- Leave the cells in the treatment media for 24 hours
- Collect supernatant after 24 hours and store in in the glass vials
- Store the samples at 4 °C until the point of steroid extraction

**Adapted from Magoffin and Erickson (1988).

ADDENDUM D**SOP: Steroid Extractions for A4 and T**

- Make up Standards

STANDARD	CULTURE MEDIA (μL)	STEROID MIX (1000ng/ML)	CONCENTRATION (ng/ ML)
S ₀	500	0	0
S ₁	500	0.5	1
S ₂	500	2.5	5
S ₃	500	5	10
S ₄	500	12.5	25
S ₅	500	25	50
S ₇	500	50	100
S ₈	500	125	250

MTBE extraction Method

- Add internal standard to the sample BEFORE adding MTBE (15ng per sample to 150 μL methanol, you want 1ng sample at the end)
- Prepare the calibration or standard series per sample batch. Standards are made in the same way as the samples to limit the error in analysis. Make up the desired concentration of media and add the media : steroid to MTBE in a 1:3 ratio. When working up, remember to add internal standard (1ng/mL sample)
- Add 3mL MTBE for each mL of cell media
- Mix and vortex the samples
- Freeze (-80 °C) until the samples have until there are two distinctive layers
- Transfer the clear layer to a clean test tube
- Evaporate the MTBE with a N₂ stream
- Reconstitute in 300 μl of 50% methanol (LCMS grade)
- Add to 150 μl the CMS vials and store at -20°C until samples are evaluated

ADDENDUM E**SOP: Steroid Extractions - Estrogens**

- Make up Standards

STANDARD	CULTURE MEDIA (μL)	STEROID MIX (1000ng/ml)	STEROID MIX (1ng/ml)	CONCENTRATION (ng/ ml)
S ₀	500		0	0
S ₁	500		5	0.01
S ₂	500		25	0.05
S ₃	500		50	0.1
S ₄	500		125	0.25
S ₅	500	0.25		0.5
S ₆	500	0.5		1
S ₇	500	2.5		5
S ₈	500	5		10
S ₉	500	12.5		25
S ₁₀	500	25		50
S ₁₁	500	50		100

MTBE extraction Method

- Add internal standard to the sample BEFORE adding MTBE (15ng per sample to 150 μL methanol, you want 1ng sample at the end)
- Prepare the calibration or standard series per sample batch. Standards are made in the same way as the samples to limit the error in analysis. Make up the desired concentration of media and add the media: steroid to MTBE in a 1:3 ratio. When working up, remember to add internal standard (1ng/ml sample)
- Add 3mL MTBE for each mL of cell media
- Mix and vortex the samples
- Freeze (- 80 °C) until the samples have split
- Transfer the clear layer to a clean test tube
- Evaporate the MTBE with a N₂ stream
- Resuspend the dried samples in 100 μL of 0.1 M Na₂CO₃
- Add 100 μL dansyl chloride solution (2 mg/ml in acetone) to each sample
- Store samples at - 20 °C - samples must be analysed within 18 hours of the dansyl chloride addition (prevents sample degradation)