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EFFECTS OF CLOMIPHENE CITRATE ON THE EXPRESSION OF KISSPEPTIN, DYNORPHIN A AND NEUROKININ B IN FEMALE SPRAGUE-DAWLEY RATS

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A dissertation in fulfillment of the degree of Magister Scientiae in Human Physiology, Faculty of Health Sciences, at the University of Pretoria

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Effects of clomiphene citrate on the expression of kisspeptin, dynorphin A and neurokinin B in female Sprague-Dawley rats

C. FOURIE

KEYWORDS

Clomiphene citrate

KNDy neurons

Kisspeptin

RT-PCR

ELISA

p234-penetratin

Ovulation induction

SERM

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ABSTRACT

Clomiphene citrate (CC) is the leading treatment for women with anovulatory infertility. The precise mechanism of action of the drug on the hypothalamic-pituitary-gonadal (HPG) axis has yet to be determined. Neurons expressing kisspeptin, neurokinin B (NKB) and dynorphin A (Dyn), collectively called KNDy neurons, in the arcuate nucleus (ARC) of the hypothalamus have been shown to play an integral role in the estradiol (E2) feedback pathways of the reproductive system in mammals. KNDy neurons are found in the ARC and the anteroventral periventricular nucleus (AVPV) in humans but have been predominantly reported to not express NKB and Dyn in rodents. The axons of these neurons project to the medial eminence (ME) in the region where the gonadotropin-releasing hormone (GnRH) terminals and fibres are located. It was hypothesised that CC upregulates the gene expression of kisspeptin and neurokinin B while down-regulating the gene expression of dynorphin A which results in a leutenizing hormone surge and an increase in oestradiol which causes ovulation.

This was a randomized experiment which included 18 female Sprague-Dawley rats in which the aim was to analyse the expression of kisspeptin, NKB and Dyn in the ARC and the AVPV as well as blood plasma levels of oestradiol and leutinizing hormone (LH) in female rats after CC administration. Six of the rats constituted the control group that received a vehicle solution. The second group of 6 rats received the intervention in the form of CC and the third group of six rats received CC as well as p234-penetratin, a kisspeptin antagonist (KpA). The mRNA expression of the KNDy genes were analysed using real-time quantitative polymerase chain reaction (qPCR) and the plasma levels of E2 and LH were analysed by enzyme-linked immunosorbant assays (ELISA).

ELISA results show that the E2 concentration in the group that received CC plus KpA was found to be marginally lower than that of the control group but there was no significant difference between the E2 concentrations of the control group and the group that received only CC. The LH concentration in the group that received CC plus KpA was significantly higher than both other groups but again, there was no significant difference between the LH concentration control group and the group that only received CC.

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The qPCR showed that in the AVPV, the kisspeptin expression of the CC group and the CC plus KpA groups are marginally higher than that of the control group. Conversely, the Dyn expression of the CC group and the CC plus KpA groups are marginally lower than that of the control group in the AVPV. There were no significant differences in NKB expression across the three groups. In the ARC, there were no significant differences in kisspeptin or Dyn expression across the groups. The NKB expression of the CC group was marginally lower than that of the control and there was no significant difference between the CC plus KpA group and the control group.

In summary, CC appears to have a marginal effect on the kisspeptin and Dyn mRNA via the positive feedback systems in the rat AVPV as well as a significant decrease of NKB mRNA via the negative feedback systems in the ARC. To increase the validity of similar future studies, higher sample sizes, different drug administration doses, possibly more precise surgical techniques and more accurate age determination methods or ovariectomised rats could be used.

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DEDICATION

This thesis is dedicated to my loving husband, Pieter Taljaard, to my parents, Pieter Fourie and Elna Fourie, and the rest of my family and friends for their endless love, support and encouragement.

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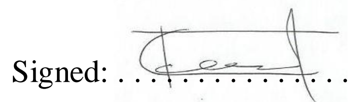
DECLARATION

I declare that the thesis Effects of clomiphene citrate on the expression of kisspeptin, dynorphin A and neurokinin B in female Sprague-Dawley rats is my own work, that it has not been submitted before for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged as complete references.

Signed:  1 November 2016

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LIST OF ABBREVIATIONS

AI	Aromatase Inhibitors
ARC	Arcuate nucleus
AUCC	Animal Use and Care Committee
AVPN	Anteroventral periventricular nucleus
CBX7	Chromobox homolog 7
CC	Clomiphene citrate
CNS	Central nervous system
COA	Chronic oligo-anovulation
dUTP	Deoxyuridine triphosphate
Dyn	Dynorphin A
E2	Oestradiol
EED	Embryonic ectoderm development
ER	Oestrogen receptors
ER-α	Oestrogen receptor alpha
ER-β	Oestrogen receptor beta
FSH	Follicle stimulating hormone
GnRH	Gonadotropin-releasing hormone
GPCR	G-protein-coupled receptors
GPR54	G-coupled receptor 54
HPG	Hypothalamus-pituitary-gonadal
IUI	Intrauterine insemination
IVF	In vitro fertilisation
Kiss1	Kisspeptin gene

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Kiss1r	Kisspeptin receptor gene in rodents
KISS1R	Kisspeptin receptor gene in humans
KNDy	Network of kisspeptin, neurokinin B and dynorphin A neurons
KpA	Kisspeptin antagonist
KOR	κ-opioid receptor
LH	Luteinising hormone
ME	Medial eminence
nIHH	Normosmic idiopathic hypogonadotropic hypogonadism
NK3	Neurokinin B gene
NK3R	Neaurokinin B receptor gene
NKB	Neurokinin B
NRT	No reverse transcription control
NTC	No template control
PcG	Polycomb group
PCOS	Polycystic ovarian syndrome
PCR	Polymerase chain reaction
POA	Preoptic area
POL II	Polymerase II
qPCR	Real-time qualitative polymerase chain reaction
SCN	Suprachiasmatic nucleus
SERM	Selective oestrogen receptor modulators
Tacr3	Tachykinin receptor 3
VIP	Vasoactive intestinal peptide
WHO	World Health Organisation
YY1	YY1 transcription factor

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LITERATURE REVIEW

1.1. Reproduction is dependent on CNS activity via the HPG Axis

The central nervous system plays a pivotal role in the various functional pathways involved in reproduction in all mammal species. These pathways are controlled by the interaction of neurons, hormones and neuromodulators. The backbone of reproduction can be explained in terms of the hypothalamic-pituitary-gonadal axis as shown in Figure 1. In response to internal and external stimuli, including positive and negative feedback signals, the hypothalamic GnRH neurons in the medial eminence of the brain secrete GnRH which, in turn, causes the anterior pituitary to secrete the gonadotropins, namely LH and follicle stimulating hormone (FSH)[1–5].

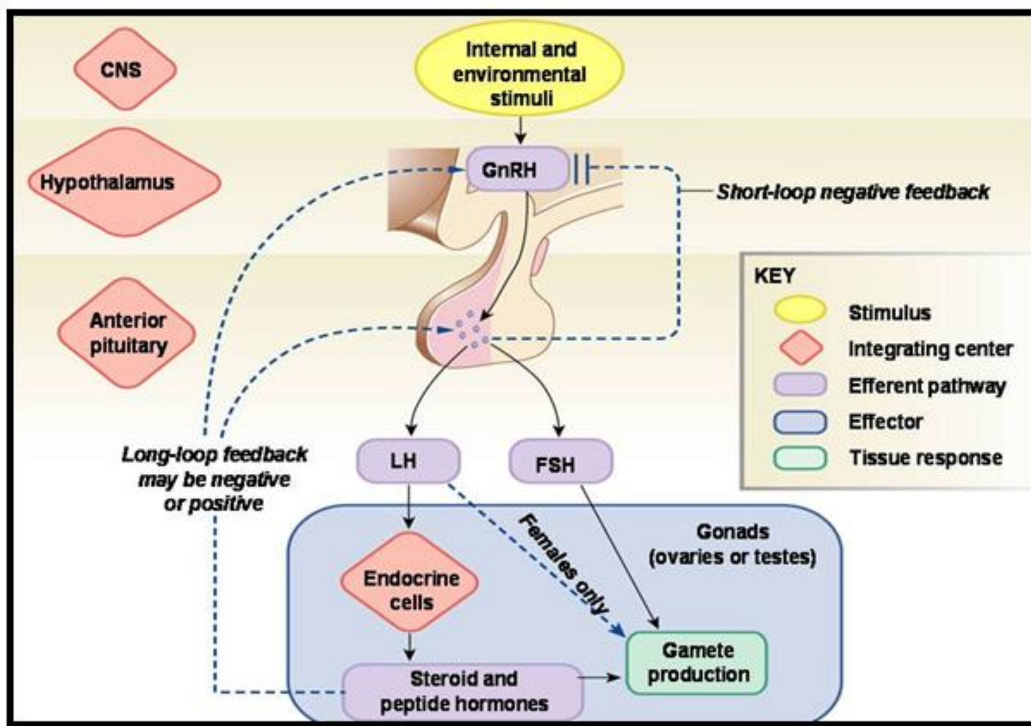

Figure 1: The HPG Axis and its feedback pathways[1].

In the female, LH causes ovarian endocrine cells to secrete steroid hormones including progesterone and androgens which are then converted to oestrogens by aromatase enzymes in the

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granulosa cells of the ovaries. Various types of oestrogens exist with the most common one, E2, found in women of child-bearing age[6]. The ovarian endocrine cells also secrete peptide hormones like inhibin and activin which respectively cause inhibition and activation of FSH secretion. FSH promotes follicle maturation during gamete formation. LH, FSH and the various sex hormones therefore work in unison to promote gametogenesis in the ovaries[1].

As shown in Figure 1, various feedback pathways control this process. Gonadotropins inhibit the release of GnRH at the hypothalamic level. Progesterone exhibits a strong negative feedback effect on GnRH release. Oestradiol's activity alternates between positive and negative feedback activity. Throughout the majority of the menstrual cycle, low oestradiol levels inhibit GnRH release via negative feedback. However at mid-cycle, when the oestradiol levels are above a certain threshold for more than 36 hours, it creates a positive feedback system where the release of the gonadotropins is stimulated, which in turn causes the production of more oestrogens and stimulates gametogenesis. GnRH is released in a pulsatile manner every one to three hours and ovulation is triggered by subsequent LH and FSH surges. When oestradiol levels are above threshold, it causes up-regulation at the level of GnRH release which, in turn, will cause very high levels of LH and relatively high levels of FSH to be present in the ovaries. FSH levels will not be as high as LH levels because of the negative feedback loops of inhibin and oestrogens as shown in **Error! Reference source not found.**. This LH surge is essential to ovulation and precedes ovulation by sixteen to twenty-four hours. It can therefore be deduced that the stimulation of hypothalamic GnRH release which in turn causes the LH surge is responsible for the induction of the ovulation process[1–5].

In females, there are two stages of GnRH and subsequent LH secretion and they occur at different times during the ovarian cycle. Tonic GnRH/LH secretion occurs in an episodic fashion throughout most of the cycle. This stage is controlled by the negative feedback actions of sex steroids, including oestradiol inhibiting pulse amplitude and progesterone inhibiting pulse frequency. The GnRH and subsequent LH surge that occurs at the end of the follicular phase prior to ovulation is induced by the positive feedback actions of high oestradiol concentrations[7].

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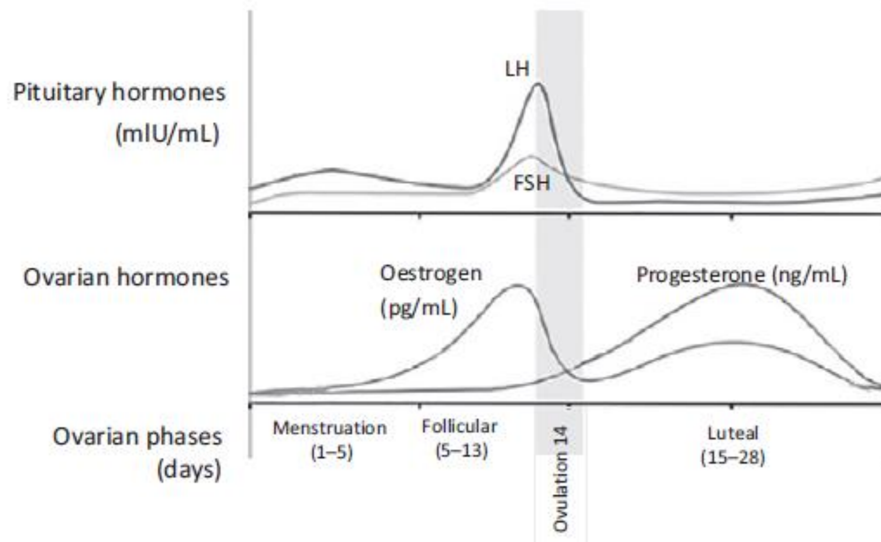

Figure 2: Plasma hormone concentration during a typical ovulatory menstrual cycle

1.2. Hypothalamic Neurons That Influence GnRH Release: The KNDy Neurons

1.2.1. General Overview

In 2003, three different groups of researchers in different areas of the world discovered that a ligand called kisspeptin and its receptor, KISS1R (previously named GPR54) played an integral role in sexual maturation and that kisspeptin may influence both positive and negative sex steroid feedback systems in the hypothalamus via GnRH-induced gonadotropin release[8–10]. Seminara et al[10] as well as de Roux et al[8] simultaneously observed that patients with idiopathic hypogonadotropic hypogonadism (IHH), a condition of failed sexual development, dysfunctional gamete production as well as infertility, had inactivating mutations or deletions of the KISS1R gene[11,12]. Simultaneously, Funes et al[9] observed that Kiss1r gene knockout mice also displayed hypogonadotropic hypogonadism indicating a parallel physiological system to the human one observed by Seminara et al and de Roux et al. Since these genetic revelations were made, the exact pathway and mechanisms of the influence of kisspeptin and its receptor on reproductive function has been thoroughly investigated by researchers from various disciplines[11].

Kisspeptin neurons in the ARC of the hypothalamus co-express NKB and Dyn[13]. Together, these are referred to as KNDy neurons which are strongly conserved across several species ranging from rodents to humans and play a pivotal role in the physiological control of GnRH neuron

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activity[7,14,15]. The axons of these neurons project to one another and they also project to the median eminence in the region where the GnRH terminals and fibres are located as shown in **Error! Reference source not found.**. Kisspeptin, NKB and Dyn act together as neuromodulators[15,16].

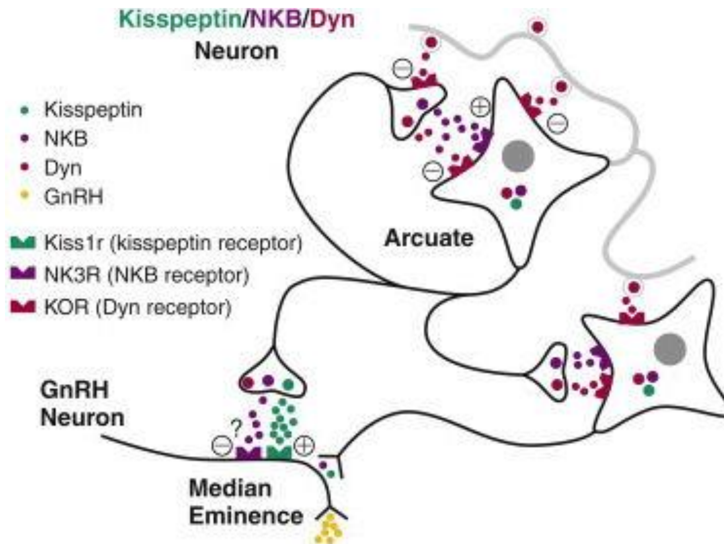

Figure 3: The interaction between KNDy and GnRH neurons[68]

As mentioned above, ovulation is caused by the LH surge from the anterior pituitary gland. This rise in LH levels causes the production of oestradiol which inhibits GnRH secretion from the hypothalamus via a hormonal feedback system of which the exact details have not yet been empirically determined. Historically, it was believed that GnRH neurons do not contain oestrogen receptors (ERs)[17]. Therefore it has been inferred that there must be an intermediary neuron in this feedback loop. It was subsequently discovered that kisspeptin neurons contain ER-α, previously found to be the predominant ER involved in oestrogen negative feedback control on GnRH function[17]. ER-α has been shown to be expressed in at least 60% of kisspeptin neurons in the male mouse brain, particularly in the AVPV. Similarly, ER-α has been shown to be expressed in over 90% of the corresponding kisspeptin neurons in the female mouse brain. It was therefore inferred that kisspeptin neurons are the intermediate step in the oestrogen-GnRH negative feedback loop[15,16,18–20]. Recently it was found that GnRH neurons do contain ER-β, which has not been found to be involved in oestrogen-GnRH feedback systems[17,21].

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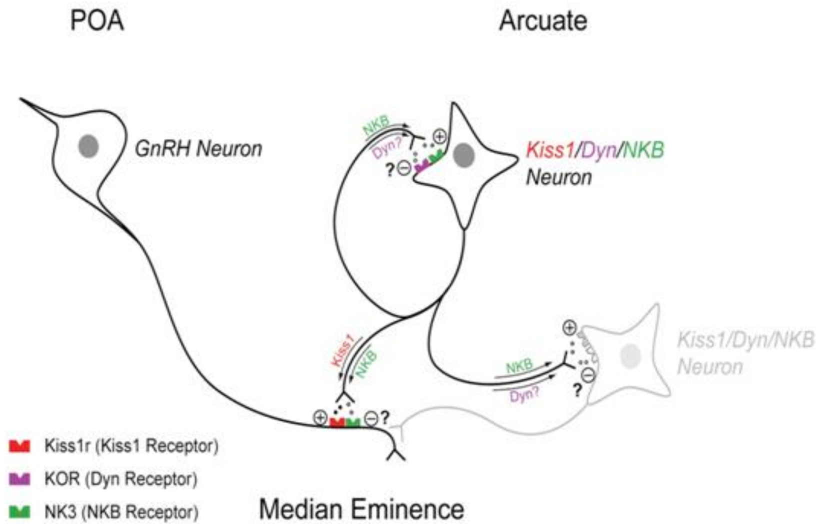

***Figure 4: Schematic diagram of the role of KNDy neurons in the hypothalamus. This system is also named the "GnRH pulse regulator". The faded neuron represents the action of the KNDy neuron when no oestradiol is present*[15].**

Research shows that there are two types of neuronal populations of kisspeptin in the rodent brain. As mentioned above, the kisspeptin population in the ARC is coexpressed with Dyn and NKB. According to immunohistochemistry studies, E2 is believed to inhibit KNDy neurons in the ARC and to increase kisspeptin expression in the AVPV[22].

In the ARC, NKB and Dyn act autosynaptically and parasynaptically on the KNDy neurons as illustrated in Figure 4. NKB up-regulates the KNDy neuron's activity whereas Dyn down-regulates it. Furthermore, kisspeptin up-regulates the GnRH neuron activity, whereas NKB down-regulates GnRH neuron activity in the ME[23]. Immunohistochemistry and PCR studies have shown the presence of KNDy neurons in the ARC but not in the AVPV[24].

Mutations in the genes that encode for kisspeptin and NKB neurons as well as their respective receptors result in an individual with IHH. This suggests that the combined actions of these two components are essential for the normal development and functioning of the reproductive system[12,25–28].

1.2.2. Kisspeptin

In 1996, the gene named Kiss1 was found to be a metastasis suppressor gene of malignant melanoma cell lines since it was shown to have powerful anti-metastatic potential. In the years

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since this gene's discovery, it has been found that it encodes a family of peptides known as kisspeptins. These arise from a common precursor which is proteolytically processed, yielding various peptides[29,30]. The precursor, 145 amino acids long, is cleaved to form a 54 amino acid residue peptide, called Kp54 or metastin[20,31,32]. Kp54 undergoes further cleavage to yield various short peptides called Kp10, Kp13, Kp14 and Kp16. These short peptides are called endogenous neuropeptides[29,33].

Shortly after the identification of the family of kisspeptin peptides, the G-protein-coupled receptor (GPCR), GPR54 was identified to be the receptor for kisspeptin peptides. Researchers found that mutations in the GPR54 gene resulted in reproductive anomalies which was important in determining kisspeptin's role in the reproductive pathways, specifically its effect on GnRH release. Subsequent to this discovery, the GPR54 receptor was renamed the KISS1R in humans and Kiss1r in rodents. During the past decade, studies have focussed on the premise that kisspeptin neurons stimulate GnRH neurons via sex steroidal feedback systems because of the fact that kisspeptin neurons express oestrogen receptors[3,23,29,30,34,35].

Kisspeptin plays an essential role in the regulation of the reproductive pathways including its negative feedback loops. When kisspeptin is centrally administered to prepubertal and adult animals, GnRH and gonadotropin release are stimulated. The stimulatory effects of kisspeptin have been shown to be directly on the GnRH neurons and not on the anterior pituitary gland as previously thought. This was shown by the fact that when kisspeptin was administered to animals together with a GnRH antagonist, acycline, no LH was released[36].

Two main neuronal populations of kisspeptin have been discovered in mammals. The first, as mentioned above, is the most prominent population of kisspeptin neurons and are expressed in the

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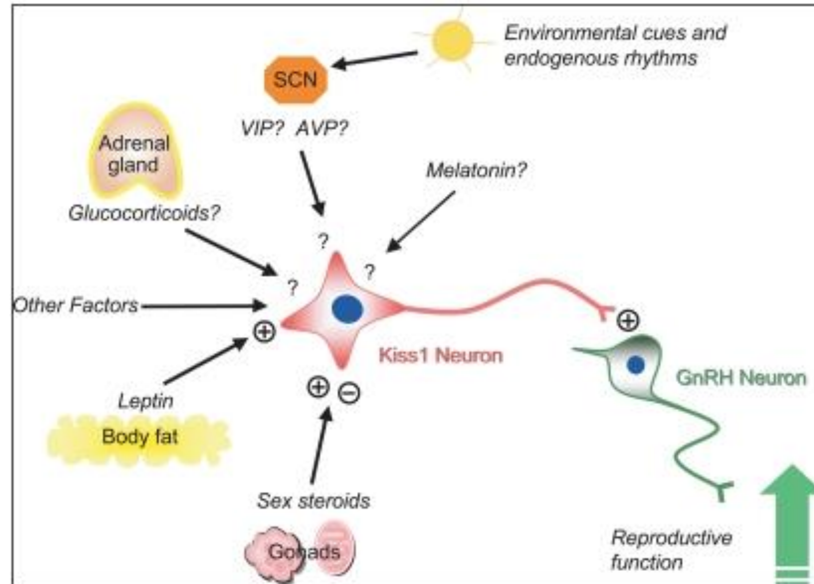

Figure 5: Kisspeptin neurons may act as central processors for relaying signals from the periphery to GnRH neurons*[37]*.

ARC[29]. In the ARC, NKB and Dyn are co-expressed with kisspeptin. The other kisspeptin population is species-dependent and has large variability with regards to its distribution in the CNS. It has been found that rodents express kisspeptin in the rostral periventricular region of the hypothalamus. This includes the AVPV. It has been noted that male rodents have much less kisspeptin neurons than females. However, the second population of kisspeptin expression in primates and sheep are found mainly in the preoptic area (POA). Even though these kisspeptin populations are situated in different locations in the brain, they all synapse with GnRH neurons[4,29,38]. The synapse between these two neuron populations stimulates GnRH release and subsequent gonadotropin release[15,29]. This concept is illustrated in Figure 5.

In rodents, the LH surge that precedes ovulation has been found to be controlled by positive oestradiol feedback loops in the hypothalamus via a "classical signalling pathway". In female rodents, Kiss1 mRNA in the AVPV is increased by oestradiol[33]. In the AVPV, kisspeptin neurons co-express α and βERs. Oestradiol stimulates kisspeptin neurons via ER-α, which has been shown to be the primary receptor influencing Kiss1 expression up-regulation[33].

It has been shown that plasma E2 increases the expression of kisspeptin in the AVPV of rodents, mediating the positive feedback of E2 on LH secretion, whereas E2 decreases kisspeptin

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expression in the ARC suggesting its involvement in the negative feedback of E2 on LH secretion[15,39].

Finally, in the ARC of rodents, kisspeptin neurons have been shown to express κ-opioid receptor (KOR) and NK3 receptors. Since these are the receptors for Dyn and NKB respectively; the kisspeptin/NKB/Dyn complex appears to be an autosynaptic feedback loop in rodents[33].

1.2.3. Neurokinin B

NKB is encoded by the Tac3 gene in rodents (TAC3 in humans) and is a signalling molecule of the tachykinin family. NKB acts on GPCRs called NK_3R (encoded by the TACR3 gene) which causes a response in target cells[13,15]. NK_3R receptors are located on KNDy neurons as well as on GnRH neurons in the ME[23,40]. Recent studies have determined that TAC3 and TACR3 gene mutations are linked to normosmic isolated hypogonadotrophic hypogonadism (nIHH), a condition of dysfunctional sexual development and gamete production as well as infertility[26,41]. This is an indication that the NKB-NK_3R complex is necessary for the normal functioning of the HPG axis and its feedback systems[25,26].

The KNDy neurons are regulated by sex steroids via ER-α. It has been determined that NKB expression in the ARC is inhibited by oestrogen, which suggests a linking role of NKB in the ARC in the negative feedback loop of the HPG axis via oestrogen. NKB up-regulates GnRH release by triggering depolarisation of KNDy neurons. It therefore has the ability to overcome Dyn's inhibitory effects on GnRH[15]. Since oestradiol causes a negative feedback loop on GnRH secretion, as oestradiol levels in the hypothalamus rise to a level below its threshold, the stimulatory signal caused by NKB binding to its receptor NK_3R is diminished. Therefore, the KNDy neurons are down-regulated which decreases the pulsatile GnRH release[23,40].

1.2.4. Dynorphin A

Dynorphin A is an endogenous opioid neuropeptide that acts on GPCR opioid receptors called κ opiod receptors (KOR). Dyn causes inhibition of cellular depolarisation which is usually induced by NKB. In recent studies, a model has been proposed where Dyn acts as the KNDy system's "brakes" when it binds to KOR on kisspeptin, NKB and GnRH neurons. It has also been found that prolonged disruption of Dyn signalling leads to continuous activation of the kisspeptin and NKB neurons, leading to lowered LH levels as a result of the kisspeptin cascade acting on GnRH

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continuously, leading to desensitisation of GnRH neurons to kisspeptin and NKB signals[7,15]. According to Lehman, Coolen et al[7] Dyn also mediates the inhibitory feedback control progesterone has on GnRH secretion.

1.3. Clomiphene Citrate

1.3.1. General Overview

Clomiphene citrate (CC) is a non-steroidal, oestrogen agonist and antagonist that has been widely used for the treatment of female anovulatory infertility caused by hypothalamic or pituitary gland dysfunction for more than forty years after being approved as an ovulation-inducing compound in 1967[43]. CC has been the cornerstone of this category of infertility treatment because it is very efficient in ovulation induction; it is inexpensive, easy to use and has relatively few serious side effects[44–46]. CC is specifically used to treat patients with a Class II ovulatory deficiency according to WHO[47].

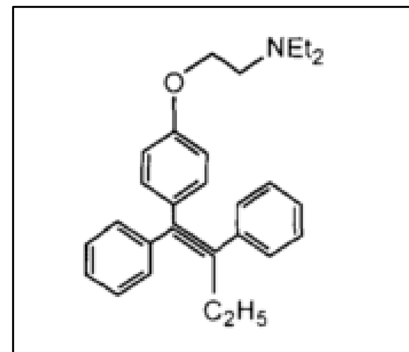

Figure 6: The chemical structure of clomiphene citrate[42].

Clomiphene has two structural isomers; cis and trans conformations. The cis formation is called zuclomiphene, which is primarily an oestrogen antagonist whereas the trans conformation, called enclomiphene is primarily an oestrogen agonist. The oestrogen antagonistic zuclomiphene's action is the main mechanism of action that results in ovulation induction during treatment with clomiphene citrate[25]. This is possible because CC is a selective oestrogen receptor modulator (SERM) and can therefore bind to oestrogen receptors and modulate their activity. Similar examples of SERMs are raloxifene and tamoxifen[45]. Climophene is a derivative of triphenylethylene and it is made up of zuclomiphene and enclomiphene salts. Zuclomiphene has been called the more influential citrate component as it has been shown to exhibit a more powerful ovulation-inducing quality. Zuclomiphene also constitutes 38% of the total drug content[43,45,47,48].

CC was initially used as a form of treatment for patients suffering from breast cancer, endometriosis and uterine hyperplasia. However, after its clear ovulation-inducing ability was

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observed, CC became a suitable treatment for infertility, together with intrauterine insemination (IUI) and in vitro fertilisation (IVF) treatments[48].

However, the main challenge in obtaining high rates of successful treatment outcomes with CC lies in the fact that the exact cellular mechanisms of action of this compound have yet to be empirically determined[43]. Another challenge is that sustained use of CC has been linked to lower pregnancy rates as well as cases of tubal ectopic pregnancy despite the fact that few side effects have been attributed to the long half-life of CC[45,49]. Zuclomiphene, the more potent citrate component of CC can be metabolized in a variety of ways which results in a complex variability in different patients' responses to the treatment[43].

1.3.2. Proposed Mechanisms of Action of Clomiphene Citrate

Even though there have been numerous advances in infertility pharmacotherapies by way of trial and error methods in the decades since CC was first discovered as an ovulation inducer, the same fundamental pharmacodynamics principle of CC as a SERM remains that it has the ability to bind to oestrogen receptors and regulate their activity via agonistic as well as antagonistic mechanisms[45].

When CC is bound to an ER, the CC-ER complex brings about oestrogen antagonistic action by regulating the target tissue's transcriptional activation[43]. CC has the ability to cause inhibition when it competes with oestrogen for binding sites on ERs. It is important to note that the CC-ER complex stays bound for longer than the oestrogen-ER complex, especially α-ERs. This leads to the inhibition of the negative feedback potential that the oestrogens possess[34,49,50].

Since ovulation is preceded by folliculogenesis caused by the combined actions of FSH and LH and these gonadotropins' release is stimulated by the release of GnRH, it is proposed that the mechanism of action of CC would include stimulation of LH and FSH, specifically the LH surge via the GnRH pathway in order to bring about the ovulation process[49]. It is hypothesized that CC influences GnRH release amplitude as well as frequency by inhibiting the oestrogen-sensitive negative feedback action on ER resulting in the subsequent increase of LH and FSH secretion which causes the activation of the ovulation process[34,49]. Further evidence for this hypothesis is observed in occurrences when CC is administered within two to three days of the follicular phase

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of the ovulation cycle. This has been shown to cause an increase in LH pulse frequency. It can therefore be deduced that CC causes the secretion of GnRH from the hypothalamus, which results in an increase in plasma LH and FSH concentration and subsequent increase in plasma oestrogen concentration prior to ovulation[45,49].

It has also been hypothesised that enclomiphene, the trans conformation of clomiphene has oestrogen agonistic effects on the anterior pituitary gland, increasing its sensitivity to GnRH from the hypothalamus, therefore increasing the release of the gonadotropins[50]. Clomiphene has also been shown to have oestrogen agonistic effects on the granulosa cells of the ovaries, increasing their sensitivity to LH and FSH. This also results in increased aromatase activity which causes increased oestrogen secretion[43,50].

In the current study, it is hypothesised that clomiphene citrate upregulates kisspeptin expression in the AVPV and ARC of the female rat by having an antagonistic effect on the negative feedback potential of oestrogen on the ERs on kisspeptin neurons.

1.3.3. Adverse Effects of Clomiphene Citrate

Some of the adverse effects of treatment with CC that have been recorded include various effects on endometrial and tubal function, cervical mucus changes as well as premature or excessive LH surges. An increase in the risk of developing ovarian cysts and ovarian cancer has also been observed. These effects can all be attributed to the oestrogen antagonistic effects of zuclomiphene[51]. The structure of clomiphene is also related to that of diethylstilboestrol, which has been shown to have a causative effect on urogenital abnormalities, hypospadias and testicular cancer in men born from women who were previously exposed to this compound[45]. These possible adverse effects may be due to the long half-life of CC[50]. Other rarely observed side effects include miscarriages, multiple pregnancies and ovarian cysts[51]. Although clomiphene results in an ovulation rate of 73% in anovulatory women with hypothalamic-pituitary dysfunction, it only results in a pregnancy rate of 36%. This can be explained by the oestrogen antagonistic effects this SERM has on the cervical mucus and endometrium as well as the possibility of LH over-secretion[43,50–52]. This causes impairment on the implantation, sperm transportation as well as embryonic development[53]. During successful treatment with clomiphene, LH and FSH levels rise for a period longer than during natural ovulation and this prolonged rise in FSH causes multiple

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follicles to grow and subsequently results in multiple pregnancies in several cases[54]. According to the available literature, 50–75% of patients suffering from chronic oligo-anovulation (COA) actually succeeded in ovulating after treatment with CC[55]..

1.3.4. Clomiphene Citrate Treatment Regimen

Ordinarily, CC is orally administered in a 50 mg/day dose for five days, starting on day 2 to 5 of the menstrual cycle in humans. If ovulation does occur after this first cycle, the 50mg/day regimen is maintained until either conception occurs or alternatively for at least six months. If this first 50 mg/day cycle of CC fails to result in ovulation, the dosage can be increased to 100 mg/day in the next cycle and even 150 mg/day in the third cycle if necessary. The maximum dosage per day is 250 mg/day because higher doses usually result in adverse effects including endometrial dysfunction[51,53]. Patients who have still failed to ovulate after CC doses of 150 mg/day for three or more cycles are said to be clomiphene resistant. Alternative treatments will be suggested by a specialist after subsequent tests have been done to improve the patient's chances of conceiving[50].

The LH surge can occur between day 5 and 12 after the last dose of CC but usually occurs at day 6 or 7. Patients are therefore encouraged to engage in sexual intercourse daily, starting on day 5 after the last dose of CC to optimize their chance of conceiving. Clinicians can monitor follicular development and confirm ovulation so as to aid in the timing of intercourse as well as to detect possible multiple follicles[50,51]

1.3.5. Alternative Treatments to CC

With respect to the release of LH and FSH from the pituitary, other SERMs like tamoxifen, droloxifene, idoxifene and toremiphene also have ER antagonistic properties and are hypothesised to have similar molecular mechanisms as CC[56,57]. Like CC, Tamoxifen is a SERM used to induce ovulation and is also commonly used as an adjuvant therapy to treat breast cancer. Unlike CC, tamoxifen produces an oestrogen agonistic effect in the vaginal mucosa and endometrium. Studies have shown, however, that both ovulation and pregnancy rates after treatment with tamoxifen are the same as treatment with clomiphene despite its oestrogen agonistic effects in the vaginal mucosa and endomentrium[58]. Tamoxifen may, however, be preferable over clomiphene for women

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suffering from corpus luteal defect, polycystic ovarian syndrome (PCOS) and abnormal cervical mucus due to it having an oestrogen agonistic effect on these tissues[42].

Aromatase inhibitors (AIs) block the action of aromatase enzymes which convert testosterone and androstenedione to oestrogens. AIs, such as the most commonly used letrozole, can induce ovulation without the anti-estrogenic side effects that clomiphene causes because they have no effect on oestrogen receptors[43]. By administering AI early in the menstrual cycle, oestrogen negative feedback is inhibited without depleting ERs. Blocking of aromatase activity will result in a dip in oestrogen production in the ovaries and the brain which blocks its negative feedback effects in the hypothalamus which then increases gonadotropin secretion and subsequent ovulation[54]. As the dominant follicle then grows, the oestrogen levels rise and once again provide the normal negative feedback to the hypothalamus which supresses FSH production and this is therefore less likely to result in multiple pregnancies[54]. Another “peripheral hypothesis” regarding the mechanisms of action of AIs is that follicle sensitivity to FSH is increased after AI administration. Due to androgens not being converted to oestrogen, intraovarian androgens may accumulate and stimulate follicular growth. Part of this hypothesis is that because of low circulating levels of oestrogen after AI treatment, ERs in the endometrium are upregulated. Once normal circulating oestrogen levels are restored, the endometrium then undergoes abnormally rapid growth[54].

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STUDY AIMS

2.1. Rationale for Study

A study investigating the site and method of action of clomiphene citrate could perhaps lead to a broader understanding of current treatments for anovulatory infertility in females.

2.2. Study Aim

The aim of this study was to analyse the expression of kisspeptin, neurokinin B and dynorphin A as well as the E2 and LH plasma levels in female rats after clomiphene citrate administration.

2.3. Study Objectives

1. To determine the expression levels of kisspeptin, neurokinin B and dynorphin A in the dissected hypothalamii of rats treated with a vehicle solution, with clomiphene citrate as well as with clomiphene citrate and p-234-penetratin using qPCR.
2. To observe whether p234-penetratin administration, a kisspeptin antagonist, to rats already treated with clomiphene citrate will diminish clomiphene citrate's action.
3. To determine the plasma LH and E2 levels in blood of rats treated with a vehicle solution, with clomiphene citrate as well as with clomiphene citrate and p-234-penetratin using ELISA.

2.4. Study Hypothesis

Clomiphene citrate administration will up-regulate kisspeptin and neurokinin B expression, down-regulate dynorphin A expression and increase LH and oestradiol plasma levels in female rats. The administration of p234-penetratin, a kisspeptin antagonist, will diminish clomiphene citrate's excitatory effects on the kisspeptin neuron, thereby reducing LH, and subsequent oestradiol levels in the blood.

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MATERIALS AND METHODS

3.1. Study Design

This study is a randomized trial using Sprague-Dawley rats. Concurrent controls were used. The rats were divided into three groups. The first group of rats constituted the control group that received a vehicle solution of 750 µl 0.9% NaCl per day between 10:00 and 11:00 via intraperitoneal injection which acted as a placebo. The second group rats received the intervention in the form of 10 mg/kg/day clomiphene citrate in 750 µl 0.9% NaCl vehicle solution via intraperitoneal injection between 10:00 and 11:00. The third group of rats received 10 mg/kg/day clomiphene citrate in 750 µl 0.9% NaCl vehicle solution between 10:00 and 11:00 as well as 3 x 100 µl injections of 5 nmol boluses of p234-penetratin, a kisspeptin antagonist via intraperitoneal route at 60 minute intervals after the clomiphene citrate administration on the last day of the study.

3.2. Study Setting

All handling and testing of the rats were performed at the Onderstepoort and Prinshof campuses of the University of Pretoria in a certified veterinarian laboratory (UPBRC). The animal experiments were approved by the Animal Ethics Committee of the University of Pretoria (Project Number: H008-14).

3.3. Study Population and Sampling

3.3.1. Study Population

Female Sprague-Dawley rats were used in this study. Inclusion criteria stated that the rats must be female and the rats must be immature at the start of the study (25 days old).

3.3.2. Sample size

A total of 18 female Sprague-Dawley rats were used in this study.

3.4. Study Procedure Involving Rat Subjects

18 female Sprague-Dawley rats were procured, supervised and taken care of by the technical staff of the UPBRC. The rats were placed in Eurostandard type III housing (155 x 266 x 425 mm with total floor area being 820 cm^2) in pairs. The rats were subjected to a relative humidity of 40% - 70%, temperatures of 22 ± 2 ºC and a 12-12 hour light/dark cycle. The housing was equipped with

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bedding in the form of wood shavings, tissue paper for nesting and inner toilet roll for hiding. Bells and marbles were provided as toys. Popcorn, Cerelac and vegetables were provided as treats.

In order to observe the oestrous cycles of the rats, vaginal smears were taken daily for three weeks. The 18 rats were randomly divided into three groups of 6 each. These three groups received treatments as shown in Table 1 below.

Group	Treatment
Group 1: Control	▪ Vehicle solution of 750 µl 0.9% NaCl per day via intraperitoneal injection
Group 2: Intervention	▪ 10 mg/kg/day Clomiphene Citrate in 750 µl 0.9% NaCl vehicle solution via intraperitoneal injection
Group 3: Intervention + kisspeptin antagonist	▪ 10 mg/kg/day Clomiphene Citrate in 750 µl 0.9% NaCl vehicle solution via intraperitoneal injection ▪ 3 x 100 µl injections of 5 nmol boluses of p234-pentratin via intraperitoneal route at 60 minute intervals on the last day of the study

Table 1: Description of treatment regimens for each test group

All rats were euthanized with an overdose of isoflurane after the last doses were administered. To minimise the effects of stress related hormones on the results, a period of less than one minute elapsed between time of handling and time of sacrifice.

For the purpose of ELISA analysis, 5 – 10 ml of blood was collected from the rats via cardiac puncture and stored in EDTA coated tubes. These samples were centrifuged for 15 minutes at 1000x g at 5 °C within 30 minutes of collection to collect plasma. Samples were stored at -20 °C. After the blood was collected, the brains were removed by dissection and flash frozen in liquid nitrogen for the purposes of qPCR analysis.

3.5. Rat hypothalamus nuclei isolation

To obtain samples of the AVPV and ARC of each rat subject, the method was followed according to Salehi[59]. The frozen rat brains were sliced using a scalpel. As shown in Figure 7 by the AA line, the diencephalon was first dissected out by an anterior coronal section, anterior to the optic chiasma, and a posterior coronal cut at the posterior border of the mammillary bodies as shown by

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cut BB in Figure 7. To isolate the ARC from the AVPV, another coronal cut was made in the centre of the optic tract rostral to infundibulum as shown by CC in Figure 7. Thereafter, a rostral division including the AVPV nucleus and a caudal division containing the ARC were made. The specific nuclei were removed using a tissue punch according to the guidelines in Figure 8.

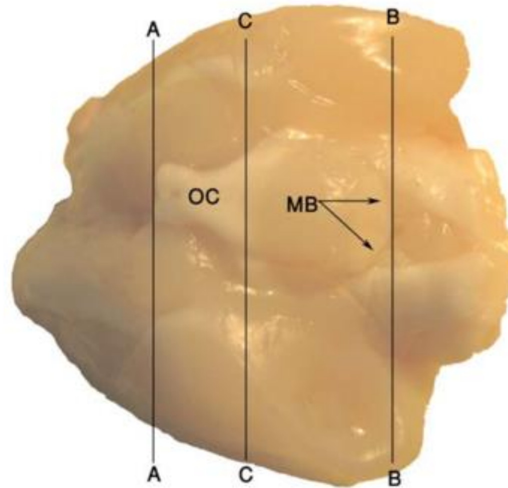

Figure 7: The ventral aspect of the rat brain. Lines A, B and C signify cutting lines[59].

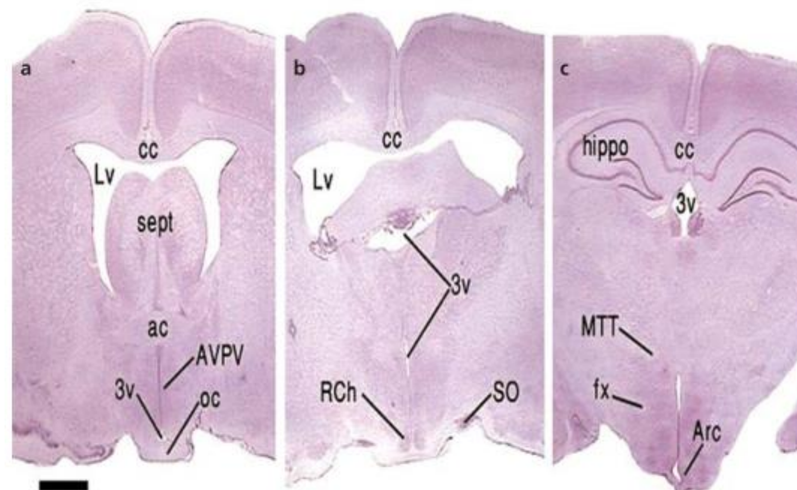

Figure 8: a) A section of the anterior division of diencephalon showing the AVPV. b) Rostral section not containing the AVPV or ARC c) Caudal section showing the ARC[59]

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3.6. Measurements – ELISA and RT-PCR

3.6.1. Enzyme-Linked Immunoabsorbent Assay

ELISA is a technique used in biochemistry which uses antibodies to evaluate substances quantitatively or qualitatively. In this study, ELISA techniques were used to quantify blood plasma levels of LH and oestradiol in female rats. The tools used in this study are called the Cusabio "Rat Oestradiol (E2) ELISA Kit"[60] and the Cusabio "Rat LH ELISA Kit"[61]. These kits use the competitive inhibition enzyme immunoassay technique. This technique uses the enzyme Horseradish Peroxidase (HRP) which, in the presence of specific substrates, converts uncoloured molecules into coloured ones to be detected and quantified by spectrophotometry at a wavelength of 450 nm. These specific ELISA kits contain a microtiter plate that has been pre-coated with goat-anti-rabbit antibodies. Standards and samples are added to the microtiter plate wells. These wells contain an antibody specific for E2/LH as well as for HRP-conjugated E2. The competitive inhibition reaction occurs between HRP-labelled E2/LH and unlabelled E2/LH with the antibody. A substrate solution is then added to the wells and the colour develops in opposite amount to that of E2/LH in the sample. After the colour development is halted the intensity of the colour can be quantified.

The precise protocol for the Cusabio "Rat Oestradiol (E2) ELISA Kit follows. Before commencing, all reagents were brought to room temperature for 30 minutes. A volume of 15 ml Wash Buffer Concentrate (20 x) was diluted with distilled water to provide 300 ml of Wash Buffer (1 x). Wells A1 to E6 were used for triplicate samples from each rat plasma sample, wells E7 to F6 were used for duplicate standards of the concentrations provided in the kit as shown in Table 2. Wells F7 to F8 were used as duplicate blank controls.

Standard	S0	S1	S2	S3	S4	S5
Concentration (pg/ml)	0	15	45	138	300	600

Table 2: Standard Concentrations for Rat E2 ELISA

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A volume of 50 µl of standards, controls and samples respectively were pipetted into each well. A volume of 50 µl of HRP-conjugate as provided was pipetted into each well except the two blank controls. Thereafter, 50 µl of given Antibody was added to each well and incubated at 37 °C for one hour. All incubation in the ELISA protocols occurred at 37 °C, away from drafts, direct sunlight and temperature fluctuations. After incubation the wells were washed three times using a wash bottle and 200 µl prepared wash buffer. After the third wash, the excess liquid and buffer in the wells were removed by inverting the well and blotting it onto paper towels. A volume of 50 µl of provided Substrate A and 50 µl of provided Substrate B were added to each well and mixed for 10 seconds. The plate was then incubated for 15 minutes. A volume of 50 µl of stop solution was added to each well and mixed for 30 seconds until the colour change was observed. The absorbance of each well was then read by a plate reader set at 450 nm.

The precise protocol for the Cusabio "Rat Oestradiol (E2) ELISA Kit follows. Before commencing, all reagents were brought to room temperature for 30 minutes. A volume of 15 ml Wash Buffer Concentrate (20 x) was diluted with distilled water to provide 300 ml of Wash Buffer (1 x). Wells A1 to E6 were used for triplicate samples from each rat plasma sample, wells E7 to F6 were used for duplicate standards of the concentrations provided in the kit as shown in. Wells F7 to F8 were used as duplicate blank controls.

Standard	S0	S1	S2	S3	S4	S5
Concentration (pg/ml)	0	0.3	1.2	4.8	15	60

Table 3 Standard Concentrations for rat LH ELISA

A volume of 50 µl of standards, controls and samples respectively were pipetted into each well. A volume of 50 µl of HRP-conjugate as provided was pipetted into each well except the two blank controls. Thereafter, 50 µl of given Antibody was added to each well and incubated at 37 °C for one hour. After incubation the wells were washed three times using a wash bottle and 200 µl prepared wash buffer. After the third wash, the excess liquid and buffer in the wells were removed by inverting the well and blotting it onto paper towels. A volume of 50 µl of provided Substrate A and 50 µl of provided Substrate B were added to each well and mixed for 10 seconds. The plate was then incubated for 15 minutes. A volume of 50 µl of the stop solution was added to each well

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and mixed for 30 seconds until the colour change was observed. The absorbance of each well was then read by a plate reader set at 450 nm.

3.6.2. Polymerase Chain Reaction

3.6.2.1. General Principles

In order to analyse the neurophysiology of the rat brain after treatment with clomiphene citrate, quantitative PCR was used by measuring the level of expression of the Kiss1, NKB and DynA genes. In this technique, each cycle of DNA amplification was monitored during the PCR process. A standard curve was then plotted and the relative level of expression of each gene was determined. Observing the relative quantities of each of these amplified genes gives an indication as to whether clomiphene citrate upregulates or down regulates the expression of these three genes or does neither.

mRNA was isolated from the rat AVPV and ARC and was then converted into DNA by reverse transcription. mRNA is produced from a processed DNA strand where introns, exons and other untranslated regions (UTRs) have been removed. The DNA resulting from the reverse transcription is called complimentary DNA (cDNA), which is not the true copy of the original strand of DNA.

A high quality cDNA preparation with minimum contamination is required for a successful PCR. Absorbance values at 260nm and 280nm are used to estimate the extracted DNA purity. A DNA sample with an A_{260}/A_{280} ratio of 1.8 to 2.0 is considered pure and ratios lower than 1.8 indicates sample contamination.

PCR uses oligonucleotide primers that are specific to a certain sequence of DNA to amplify that specific sequence. This is done by an automated system that denatures and reanneals DNA sequences, creating exact copies of the sequence of interest that can then be quantified. A long sequence of DNA containing the sequence of interest, for example the sequence that contains the Kiss1, NKB or Dyn gene, is first denatured, and then a sequence-specific primer is annealed to the single-stranded DNA (ssDNA) when the primer recognizes specific sequences. Polymerase enzymes as well as the four types of deoxynucleotides (dNTPs) are then added to the mixture and the primers are then lengthened by the polymerase action. When the dNTPs form a complimentary strand on the ssDNA, double-stranded DNA (dsDNA) is formed. This means that there is now double the amount of the sequence of interest than initially. The process is repeated in various

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cycles of denaturing and reannealing resulting in multiple copies of the sequence of interest. The Universal SYBR Green Quantitative PCR Protocol was used to quantify the amount of Kiss1, Dyn and NKB that is expressed in the hypothalamus.

Housekeeping genes like B-actin are used in quantitative PCR as an internal standard because their expression occurs in all nucleated cell types because they are necessary for basic cell survival. The expression of these genes fluctuate very little in comparison to that of others genes[62]. The reliability of a quantitative PCR experiment can be improved by including a reference gene like B-actin to correct for sample to sample variations. Analysing target mRNA copy numbers requires accurate and relevant normalisation to a housekeeping standard**.** High variation in the normalising gene could obscure real changes in data[63].

The PCR oligonucleotide sequences used in the present study were obtained from previous studies involving the specific genes involved as shown in Table 4.

Oligo-nucleotide	Sequence	Reference
Kiss Forward	5’-ATGATCTCGCTGGCTTCTTGG-3’	Oride et al[64]
Kiss Reverse	5’-GGTTCACCACAGGTGCCATTTT-3’	Oride et al[64]
NKB Forward	5’-GAGAGATCCCAGGAGACA-3’	Cintado et al[65]
NKB Reverse	5’-TGGGGTCAAACAGCACGG-3’	Cintado et al[65]
Dyn Forward	5’-CGCAAATACCCCAAGAGGAG-3’	Pravetoni et al[66]
Dyn Reverse	5’-GCAGGAAGCCCCCATAGC-3’	Pravetoni et al[66]
B-actin Forward	5’-CCTAGCACCATGAAGATCAA-3’	Cintado et al[65]
B-actin Reverse	5’-TTTCTGCGCAAGTTAGGTTTT-3’	Cintado et al[65]

Table 4: Oligo-nucleotide primer sequences for PRC

3.6.2.2. Kappa Sybr Fast One Step qRT-PCR Protocol

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Step 1: qPCR Reaction Setup

This step involves the cDNA synthesis. Before preparing qRT-PCR reactions, the KAPA SYBR® FAST qPCR Master Mix (2X), KAPA RT Mix (50X) including the dNTPs, template RNA, and primers were thoroughly mixed. The template RNA input used per sample was 95 ng total RNA. A reaction cocktail was prepared to reduce pipetting errors. Equal aliquots were dispensed into reaction tubes. RNA was added to each reaction as a final step. A No Template Control (NTC) and No RT Control (NRT) were included. The NTC enables detection of contamination in the reaction components. The NRT control tests for contaminating genomic DNA in the reaction. The required volumes of each component were calculated based on Table 5.

	Final concentration	**20 µl reaction**	**10 µl reaction**
Nuclease-free water up to 20 µl		As required	1.2 µl
KAPA SYBR® FAST qPCR Master Mix (2X)	1X	10 µl	5 µl
Forward Primer (10 µM)	200 nM	0.4 µl	0.2 µl
Reverse Primer (10 µM)	200 nM	0.4 µl	0.2 µl
dUTP (10 mM) (optional)	200 nM	0.4 µl	0.2 µl
ROX High (optional)	500 nM	0.4 µl	0.2 µl
KAPA RT Mix (50X)	1X	0.4 µl	0.2 µl
Template RNA	Variable	<5 µl	2.5 µl

Table 5: Concentrations and volumes of different components added in the qPCR reaction setup

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Step 2: Plate Setup

The appropriate volume of reaction mixture was transferred to each well of a PCR tube/plate. Reaction volumes were scaled down from 20 µl to 10 µl because low volume tubes/plates were used. The reaction tube was sealed and centrifuged briefly.

Step 3: Run qPCR reaction

The cycling protocol in Table 6 was programmed.

Step	Temperature	Duration	Cycles
cDNA synthesis	42 ºC	5 min	Hold
Inactivate RT	95 ºC	2 min	Hold
Denature	95 ºC	2 sec	40
Anneal/extend	60 ºC	22 sec	
Cooling	40 ºC	10 sec	Hold

Table 6: Cycling protocol for qPCR reaction

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RESULTS

4.1. Statistical Considerations

A 95% confidence interval statistical analysis was performed in order to determine whether there is a significant relationship between the mRNA expression levels of Kiss1, Dyn and Tac3 genes and the administration of clomiphene citrate (CC) alone as well as the administration of clomiphene citrate and p-234 penetratin (CC + KpA). These two treatment groups were compared to a control group to which no treatments were administered.

4.1.1. Sample size

Financial restrictions limited the sample size to six animals per group. Although this sample size is very small, very little variation between animals within the groups were observed.

4.1.2. Data Analysis

For the ELISA, absorbance values for each group were averaged and a 4-parameter logistic curve was created to obtain the concentrations of LH and E2 in the blood. Average group blood concentrations of LH and E2 were compared using a 1-tailed T-test assuming equal variance.

For the PCR, Kiss1, Dyn and Tac3 gene threshold cycles were observed and summarised by group using mean, standard deviation, median and range. Confidence intervals (95%) were determined for the respective differences of mean threshold cycle between the CC group and CC + KpA group versus the control group. These two 95% confidence intervals were then rewritten to express 95% confidence intervals for the ratios of the CC group and the CC + KpA group relative to the control group.

Data analysis followed after Multiple Imputation (MI) with the Missing at Random (MAR) assumption so as to deal with a number of missing data points. The full raw data set with descriptive statistics can be observed in Appendix A. The imputation and statistical analysis can be observed in Appendix B.

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4.2. Results

4.2.1. ELISA Results

Immediately after the last doses of the treatments were administered on the last day of the animal procedures, the rats were euthanized with an overdose of isoflurane. 5 – 10 ml of blood was collected in EDTA coated tubes from the rats via cardiac puncture. These samples were centrifuged for 15 minutes at 1000x g at 5 °C within 30 minutes of collection to collect plasma. Samples were stored at -20 °C. A week later these plasma samples were used in the Cusabio "Rat Oestradiol (E2) ELISA Kit"[60] and the Cusabio "Rat LH ELISA Kit"[61] which yielded the following results.

4.2.1.1. E2 ELISA Results

When comparing the blood E2 concentrations between the three groups using a one tailed t-test with assumed equal variances, the following observations were made as shown in Figure 9:

1. No significance was found between the E2 concentrations between groups A and B (P-value: 0.348)
2. No significance was found between the E2 concentrations between groups C and B (P-value: 0.105)
3. The E2 concentration in group C was found to be marginally lower than that of group A but not statistically significant (P-value: 0.067).

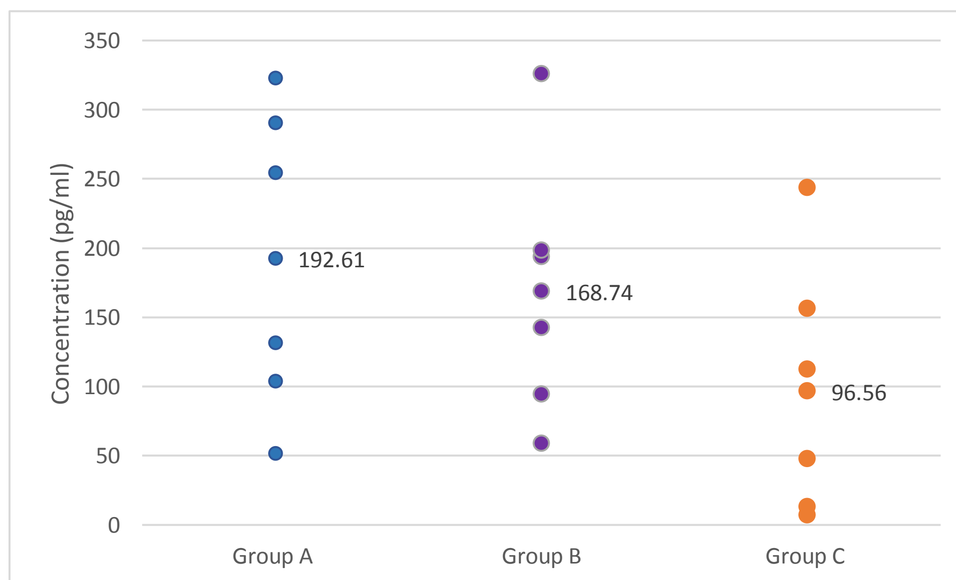

Figure 9: E2 concentration individual value plot showing group means

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4.2.1.2. LH ELISA Results

When comparing the blood LH concentrations between the three groups, the following observations were made as shown in Figure 10:

1. No significance was found between the LH concentrations between groups A and B (P-value: 0.194)
2. The LH concentration in group C was found to be significantly higher than that of group A (P-value: 0.032).
3. The LH concentration in group C was found to be marginally higher than that of group B but not statistically significant (P-value: 0.054).

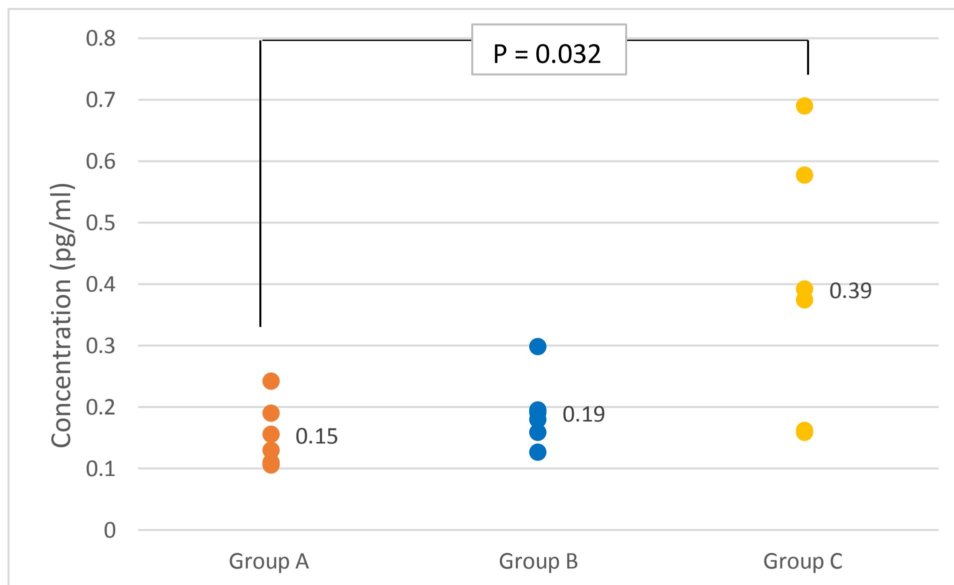

Figure 10: LH concentration individual value plot showing group means

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4.2.2. PCR results

In order to analyse the neurophysiology of the rat brain after treatment with clomiphene citrate, quantitative PCR was used by measuring the level of expression of the Kiss1, NKB and DynA genes. In this technique, each cycle of DNA amplification was monitored during the PCR process. A standard curve was then plotted and the relative level of expression of each gene was determined. These levels of expression were then normalised against the expression levels of the B-actin housekeeping gene through the ΔΔCq method[67]. In this method, expression of the target genes (kisspeptin, NKB and Dyn respectively) was normalized to non-targeted B-actin reference gene (REF) expression levels within the same sample by subtracting the B-actin threshold cycle number from the target genes threshold cycle number. By using exponential transformation and subsequent logarithmic transformation, one can determine the mean relative expression levels and standard deviations of the genes. A student T-test was then used to generate a P-value to show significance between groups.

The statistical differences in expression levels between groups are expressed as low and high percentages in relation to each other. By using 95% confidence interval calculations, one can be 95% certain that the true mean expression levels of certain genes in specific brain areas will measure at a certain range of percentages of each other as shown in the following sections. For example, if it is stated that the expression level of Group X can measure as low as 65% of the expression level of Group Y and as high as 85% of the expression level of Group Y. This means that Group Y's true mean is 100% and we can be 95% sure that group X's true mean expression is 65% to 85% of that of Group Y and is therefore lower than that of Group Y.

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4.2.2.1. Gene expression in the AVPV

4.2.2.1.1. B-actin expression in the AVPV

According to statistical analysis, the following statements can be made with 95% confidence:

1. The expression level of group B can measure as low as 86% of the expression level of group A and as high as 108% of the expression level of group A. The P-value is 0.473 and therefore not significant.
2. The expression level of group C can measure as low as 85% of the expression level of group A and as high as 107% of the expression level of group A. The P-value is 0.405 and therefore not significant.
3. The expression level of group C can measure as low as 87% of the expression level of group B and as high as 112% of the expression level of group B. The P-value is 0.976 and therefore not significant.
4. Thus there is no significant differences between the mean B-actin expression level of the three groups in the AVPV as shown in Figure 11.

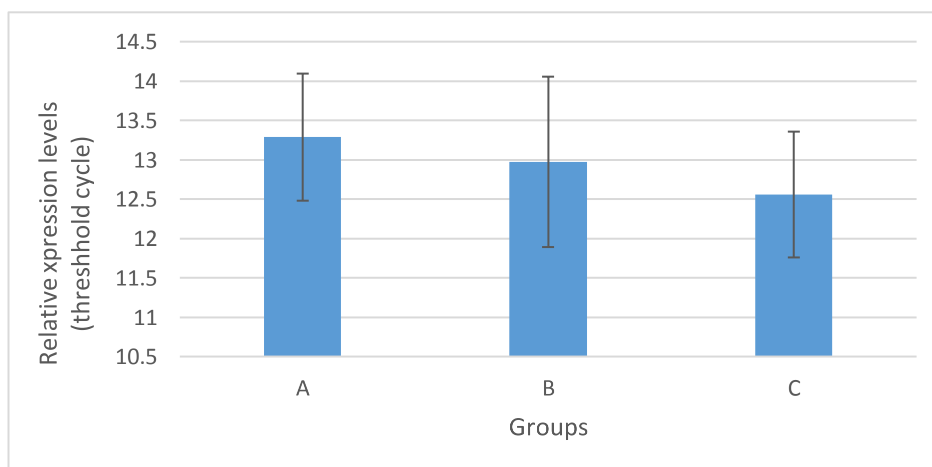

Figure 11: Mean B-actin expression in the AVPV (+/- 1 S.D.)

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4.2.2.1.2. Kisspeptin expression in the AVPV

According to statistical analysis, the following statements can be made with 95% confidence:

1. The expression level of group B can measure as low as 98% of the expression level of group A and as high as 126% of the expression level of group A. The P-value is 0.077 and therefore almost significant.
2. The expression level of group C can measure as low as 99% of the expression level of group A and as high as 141% of the expression level of group A. The P-value is 0.092 and therefore almost significant.
3. The expression level of group C can measure as low as 89% of the expression level of group B and as high as 124% of the expression level of group B. The P-value is 0.453 and therefore not significant.
4. Thus the mean kisspeptin expression of group B and group C are almost significantly higher than that of group A in the AVPV as shown in Figure 12.

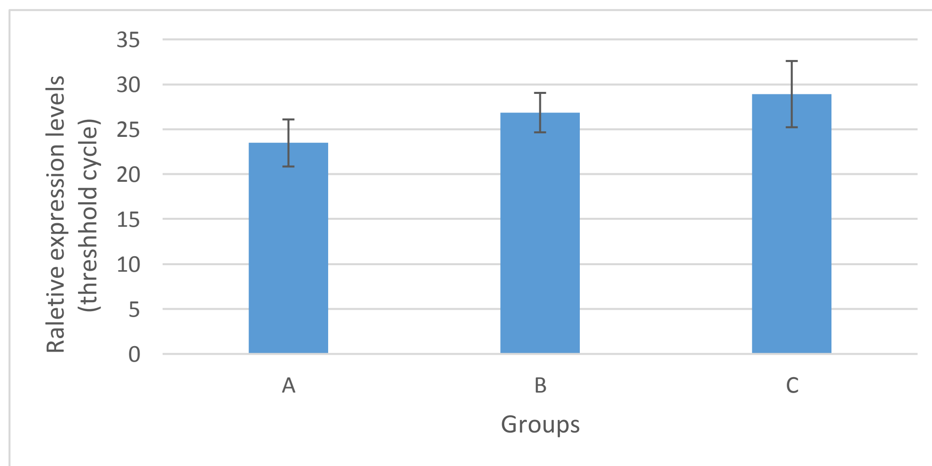

Figure 12: Mean kisspeptin expression in AVPV (+/- 1 S.D.)

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4.2.2.1.3. Dynorphin A expression in the AVPV

According to statistical analysis, the following statements can be made with 95% confidence:

1. The expression level of group B can measure as low as 83% of the expression level of group A and as high as 101% of the expression level of group A. The P-value is 0.062 and therefore almost significant.
2. The expression level of group C can measure as low as 83% of the expression level of group A and as high as 102% of the expression level of group A. The P-value is 0.071 and therefore almost significant.
3. The expression level of group C can measure as low as 90% of the expression level of group B and as high as 112% of the expression level of group B. The P-value is 0.864 and therefore not significant.
4. Thus the mean dynorphin expression of group B and group C are marginally lower than that of group A but there is no significant difference between the mean expression of group B and C in AVPV as shown in Figure 13.

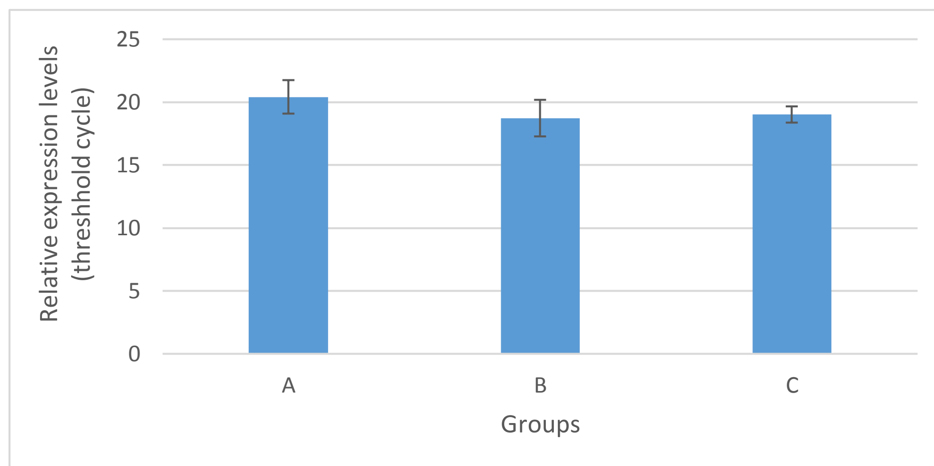

Figure 13: Mean dynorphin A expression in AVPV (+/- 1 S.D.)

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4.2.2.1.4. Neurokinin B expression in the AVPV

According to statistical analysis, the following statements can be made with 95% confidence:

1. The expression level of group B can measure as low as 73% of the expression level of group A and as high as 130% of the expression level of group A. The P-value is 0.898 and therefore not significant.

2. The expression level of group C can measure as low as 78% of the expression level of group A and as high as 165% of the expression level of group A. The P-value is 0.279 and therefore not significant.
3. The expression level of group C can measure as low as 71% of the expression level of group B and as high as 168% of the expression level of group B. The P-value is 0.399 and therefore not significant.
4. Thus there is no significant differences between the mean neurokinin B expression of the three groups in the AVPV as shown in Figure 14.

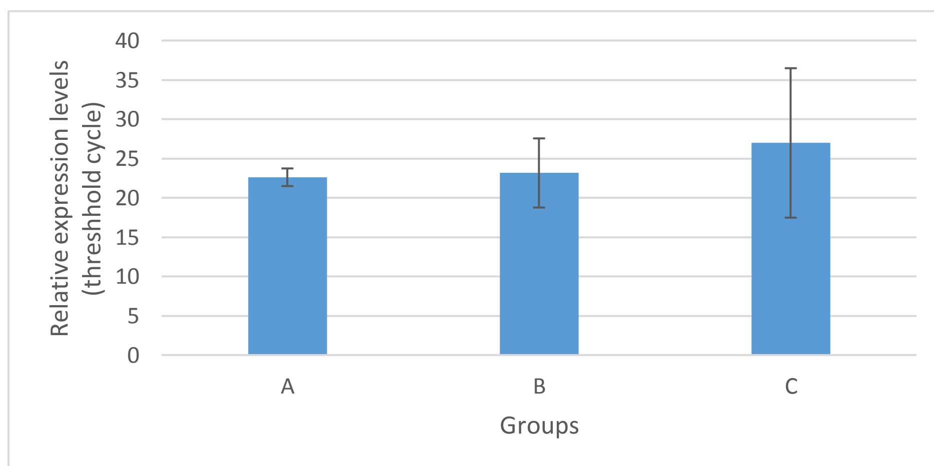

Figure 14: Mean Neurokinin B expression in the AVPV (+/- 1 S.D.)

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4.2.2.2. Gene expression in the arcuate nucleus

4.2.2.2.1. B-actin expression in the ARC

According to statistical analysis, the following statements can be made with 95% confidence:

1. The expression level of group B can measure as low as 92% of the expression level of group A and as high as 105% of the expression level of group A. The P-value is 0.417 and therefore not significant.
2. The expression level of group C can measure as low as 91% of the expression level of group A and as high as 106% of the expression level of group A. The P-value is 0.664 and therefore not significant.
3. The expression level of group C can measure as low as 92% of the expression level of group B and as high as 108% of the expression level of group B. The P-value is 0.750 and therefore not significant.
4. Thus there is no significant differences between the mean B-actin expression of the three groups in the ARC as shown in Figure 15.

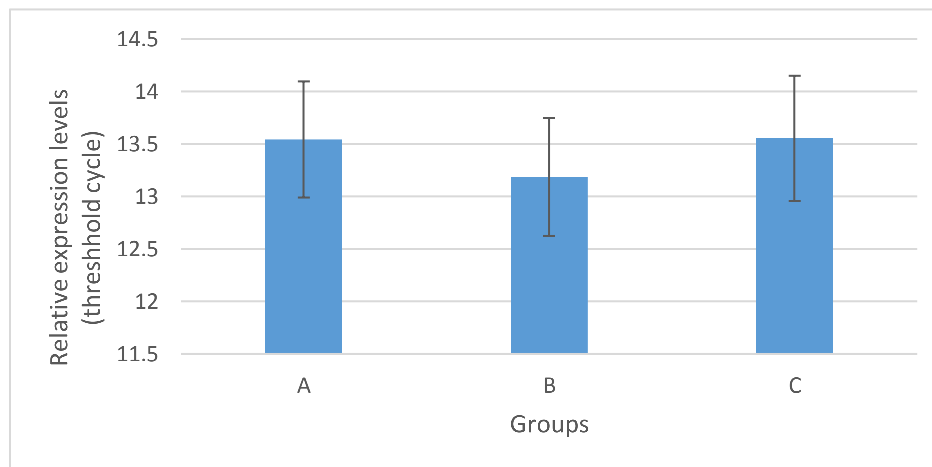

Figure 15: Mean B-actin expression in the arcuate nucleus (+/- 1 S.D.)

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4.2.2.2.2. Kisspeptin expression in the ARC

According to statistical analysis, the following statements can be made with 95% confidence:

1. The expression level of group B can measure as low as 94% of the expression level of group A and as high as 109% of the expression level of group A. The P-value is 0.653 and therefore not significant.
2. The expression level of group C can measure as low as 94% of the expression level of group A and as high as 106% of the expression level of group A. The P-value is 0.955 and therefore not significant.
3. The expression level of group C can measure as low as 93% of the expression level of group B and as high as 104% of the expression level of group B. The P-value is 0.509 and therefore not significant.
4. Thus there are no significant differences between the mean kisspeptin expression of the three groups in the ARC as shown in Figure 16.

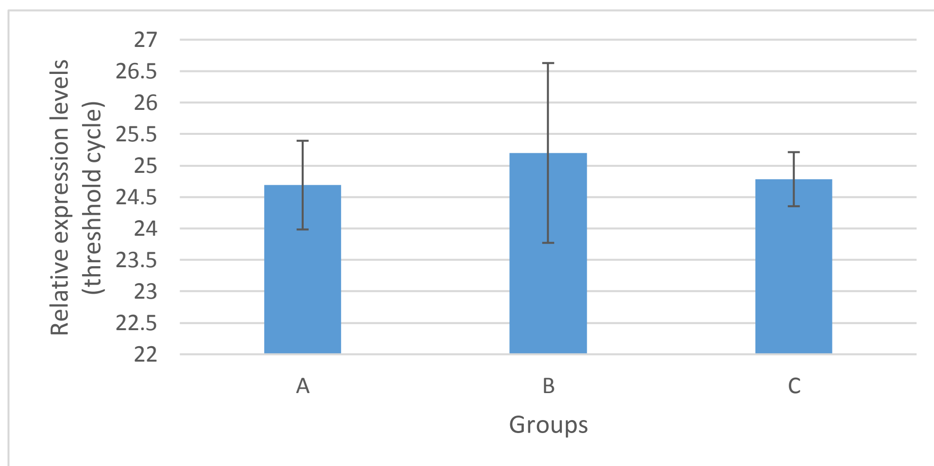

Figure 16: Mean kisspeptin expression in arcuate nucleus (+/- 1 S.D.)

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4.2.2.2.3. Dynorphin A expression in the ARC

According to statistical analysis, the following statements can be made with 95% confidence:

1. The expression level of group B can measure as low as 80% of the expression level of group A and as high as 109% of the expression level of group A. The P-value is 0.268 and therefore not significant.
2. The expression level of group C can measure as low as 83% of the expression level of group A and as high as 112% of the expression level of group A. The P-value is 0.567 and therefore not significant.
3. The expression level of group C can measure as low as 95% of the expression level of group B and as high as 111% of the expression level of group B. The P-value is 0.381 and therefore not significant.
4. Thus there is no significant differences between the mean dynorphin A expression of the three groups in the ARC as shown in Figure 17.

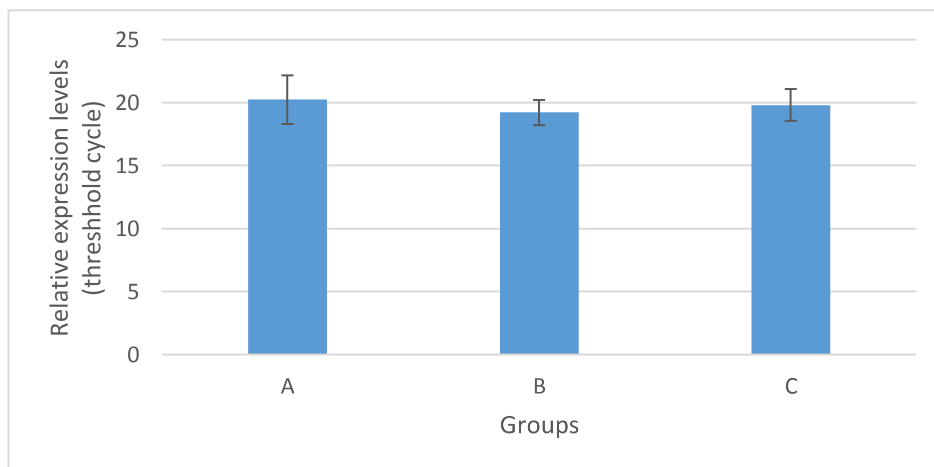

Figure 17: Mean dynorphin A expression in the arcuate nucleus (+/- 1 S.D.)

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4.2.2.2.4. Neurokinin B expression in the ARC

According to statistical analysis, the following statements can be made with 95% confidence:

1. The expression level of group B can measure as low as 82% of the expression level of group A and as high as 101% of the expression level of group A. The P-value is 0.062 and therefore almost significant.
2. The expression level of group C can measure as low as 77% of the expression level of group A and as high as 118% of the expression level of group A. The P-value is 0.656 and therefore not significant.
3. The expression level of group C can measure as low as 87% of the expression level of group B and as high as 124% of the expression level of group B. The P-value is 0.405 and therefore not significant.
4. Thus in the ARC the expression of neurokinin B of group B is significantly higher than group A and there is no significant difference between groups C and A or groups C and B as shown in Figure 18.

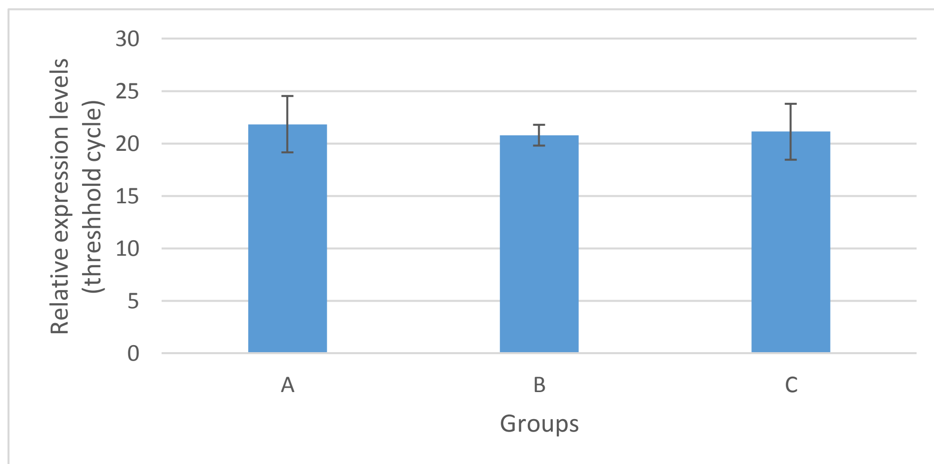

Figure 18: Mean neurokinin B expression in the arcuate nucleus (+/- 1 S.D.)

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DISCUSSION

5.1. Plasma hormone concentrations

5.1.1. Oestradiol and leutenizing hormone concentrations

As discussed in the literature review, when oestradiol levels are above threshold, it causes up-regulation at the level of GnRH release which, in turn, will cause very high levels of LH to be present and in the blood. This LH surge is essential for ovulation and precedes ovulation by sixteen to twenty-four hours. It can therefore be deduced that the stimulation of hypothalamic GnRH release which in turn causes the LH surge is responsible for the induction of the ovulation[1–5]. During ovulation, E2 levels drop significantly[68]. It can be hypothesized that if the rats in the present study are ovulating, their E2 levels would be lower than that of the rats who are not ovulating. Results showed that compared to the control, the E2 levels of group B were lower, albeit not significantly. The E2 levels of group C were marginally lower than that of group A. This suggests that the kisspeptin antagonist may have played a role in stimulating ovulation instead of counteracting it as hypothesized.

In anovulatory females, there will be no measurable LH surge.[69]. Thus it can be predicted that in the present study, the LH concentration of groups A and C would be lower than that of group B, given the CC was acting as indicated, causing ovulation. Results show that LH was increased in group B but not significantly. However, similarly to the E2 results, the concentration of group C was significantly higher than in groups A and B, indicating that the kisspeptin antagonist could have played a role in the LH surge causing ovulation.

The present study made use of the p234-penetratin kisspeptin antagonist which has been shown to be an effective antagonist in a large number of animal studies[20,70–75]. However, it has some agonist activity at high levels and has also been found to be ineffective in some animal studies[72,75]. In the present study the kisspeptin antagonist seems to be propagating the effect of CC instead of inhibiting its effects as hypothesized as together with CC it significantly stimulated LH. However, since there was no significant effect of CC alone definite conclusions cannot be made.

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5.2. Gene expression levels of kisspeptin, NKB and Dyn

5.2.1. Gene expression levels in the AVPV

It has been shown that plasma E2 increases the expression of kisspeptin in the AVPV of rodents, mediating the positive feedback of E2 on LH secretion[39]. Since CC is a SERM and can have E2-agonistic effects on E2 receptors, it is hypothesized that CC could increase the kisspeptin expression in the AVPV. In this study the mean kisspeptin expression of group B and group C were marginally higher than that of the control group but the increase was statistically significantly.

The p-234 penetratin KpA appears to have agonistic properties opposite to what was hypothesised in this study and should be investigated further. It is possible that this could be because of the timing of administration and the uncertainty of whether there was enough time for it to influence expression before the rats were euthanised. Another test group where only the KpA was administered would have given a better indication of its actions on the gene expression.

Although immunohistochemistry studies have previously indicated that there are no KNDy neurons in the AVPV[22,24], in the present study there were clear gene expression signals of kisspeptin, Dyn and NKB which were similar in magnitude suggesting that there may be gene expression but possibly not translation of the NKB and Dyn genes in the AVPV. It is possible that this is due to an error in surgical technique precision in the isolation of the different brain nuclei. Further PCR studies on the AVPV should investigate whether there is in fact expression of the KNDy neurons in the AVPV.

In the ARC, NKB and Dyn are proposed to act autosynaptically and parasynaptically on the KNDy neurons. NKB up-regulates the KNDy neuron's activity whereas Dyn down-regulates it. A recent study suggests that KNDy neurons regulate the LH surge through inhibiting AVPV kisspeptin neurons via dynorphin[22]. In the present study, the expression of Dyn was marginally lower in groups B and C than in the control group. This suggests that CC may work by both increasing kisspeptin activity and by decreasing Dyn activity. In this study the NKB expression did increase slightly in groups B and C but there was no significant.

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5.2.2. Gene expression levels in the ARC

Plasma E2 decreases kisspeptin expression in the ARC of rodents suggesting its involvement in the negative feedback of E2 on LH secretion[39]. The KNDy neurons are regulated by sex steroids via ER-α. It has been determined that NKB expression in the ARC is inhibited by oestrogen, which suggests a linking role of NKB in the ARC in the negative feedback loop of the HPG axis via E2. NKB up-regulates GnRH release by triggering depolarisation of KNDy neurons. It therefore has the ability to overcome Dyn's inhibitory effects on GnRH[15]. Since oestradiol is part of the negative feedback loop on GnRH secretion, E2 levels in the hypothalamus rise to a level below its threshold, the stimulatory signal caused by NKB binding to its receptor NK_3R is diminished. Therefore, the KNDy neurons are down-regulated which decreases the pulsatile GnRH release[23,40]. A recent study suggests that NKB is involved in increasing GnRH/LH pulsatility preceding ovulation but that it is not essential for E2-induced positive feedback[76]. For this reason, we hypothesised an increase in kisspeptin and NKB expression and a decrease in Dyn expression in the ARC after CC administration. However, in the present study it appears that there was no significant difference in kisspeptin or Dyn gene expression across the three groups and in the group that received only CC, NKB expression was significantly decreased. This could suggest that CC plays a role in the negative feedback system in the ARC via NKB. In retrospect the present study should have had a positive control of ovariectomised rats to demonstrate the well-established rise in kisspeptin gene expression in the ARC and decrease in the AVPV. This would be a useful control for the accuracy of the dissection of the areas and that the qPCR was operating effectively. Such experiments were limited by time available for the MSc thesis.

5.3. Statistical Significance

In the present study, P values of 0,051 to 0,099 were described as being marginally significant. In a study with larger sample sizes, complete data sets and less individual variation, those values would be statistically insignificant. However, in the present study, small sample sizes, missing data and high individual variation result in a more in depth statistical analysis including multiple imputation analysis and considering trends towards significance when analysing the results. The full result set can be found in Appendix A.

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FUTURE WORK AND CONCLUSION

6.1. Study Limitations and Future Work

The major limitation in this study was that the sample size was very small and additional controls such as kisspeptin antagonist alone and a positive control of ovariectomy could not be included until proof of concept had been established. This was because of the specifications in the South African National Standard (SANS 10386-2008): "The Care and Use of Animals for Scientific Purposes". The small sample size reduces the study's statistical power and reduces the probability of obtaining statistically significant results that reflect the true effects of CC on the rodent hypothalamus[77].

Only a handful of past studies have involved CC administration to rodents therefore there was very little evidence that the method of administration and dosage were optimal for the desired effects of this study[78–81]. Future studies should involve the use of lavage or intraperitoneal injections and different dosages to determine the correct administration so as to obtain an effect similar to that of CC in humans.

Even though a request for sexually immature rat subjects was issued, was apparent that a range of weights of rats was supplied indicating that they were of different maturity. If the rats were sexually maturing or mature at the time of the study, their natural ovulation and LH surges could influence the study results. In future studies matched rats of authenticated known age should be used.

To add to the validity of a similar study, vaginal smears should be performed during the study in order to first observe whether the rats that are given CC are definitely ovulating before sacrifice occurs.

The surgical technique was not optimal for definitively locating and extracting the relevant brain areas. The use of a relevant rodent brain slicer and a dissecting microscope would be beneficial to the accuracy of the tissue isolation in further studies.

A similar future study would be more significant if immunohistochemistry could be performed in addition to qPCR. Immunohistochemistry would show the localisation of kisspeptin, NKB and

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Dyn neurons in the specific areas of the rodent brains. This will add a qualitative aspect of gene expression analysis to solidify the quantitative aspect discovered with the qPCR.

The current study only focused on the mRNA expression of the three relevant genes. Further studies could investigate the protein expression of kisspeptin, NKB and Dyn by means of immunoasay.

Another aspect that could greatly strengthen a similar future study would be to add various other test groups and controls. In the present study, a group where only kisspeptin antagonist was given could have provided additional insight into its effectiveness. New test groups for future research would include a kisspeptin antagonist being administered exclusively. Another principle would be the administration of a NKB antagonist or a GnRH antagonist alone or in combination with CC or to use ovariectomy as a positive control.

The signals of KNDy neuron expression in the present study suggests that future PCR and immunohistochemistry studies should be done to determine whether KNDy neurons are possibly expressed but not translated in the AVPV of rodents.

6.2. Conclusion

The overall aim of the study was to analyse the gene expression of kisspeptin, neurokinin B and dynorphin A as well as the E2 and LH plasma levels in female rats after clomiphene citrate administration.

Plasma LH levels were significantly higher in group C signifying the LH surge and ovulation in that group.

The mean kisspeptin expression in the AVPV of group B and group C were marginally higher than that of the control group and the expression of Dyn was marginally lower in groups B and C than in the control group. Were these to be significantly different, this would suggests that CC may work by both increasing kisspeptin activity and decreasing Dyn activity. In this study the NKB expression did increase slightly in groups B and C but there was no significant difference, showing that CC could possibly have an effect on the expression of NKB in the AVPV.

In the ARC, NKB expression was significantly decreased in the group that received only CC. This could suggest that the agonist activity of CC could play a role in the negative feedback system in

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the ARC via NKB. There was no significant difference in kisspeptin or Dyn expression across the three groups in the ARC.

In conclusion, there is some indication (but not significant) that CC may have an agonist effect on the kisspeptin -and Dyn neurons via the positive feedback systems in the rat AVPV and a significant stimulation of NKB gene expression via an antagonist effect on the negative feedback systems in the ARC.

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APPENDIX A - Raw Data with Descriptive Statistics

Group A: Control (No treatment)

AVPV

	B-actin	Kisspeptin	Dynorphin	Neurokinin
3 AV	14.442	39.814	22.755	22.811
7 AV	13.483	27.32	19.53	22.314
8 AV	12.279	22.253	19.147	21.9
11 AV	13.917	25.572	19.609	24.778
16 AV	12.834	23.849	20.476	21.809
17 AV	x	21.35	20.996	22.043
Mean	13.391	26.693	20.41883333	22.6091667
STDEV	0.856815908	6.784688585	1.330975494	1.12208973
SEM	0.383179723	2.769837516	0.54336847	0.45809121
Median	13.483	24.7105	20.0425	22.1785
Range	2.163	18.464	3.608	2.969

ARC

	B-actin	Kisspeptin	Dynorphin	Neurokinin
3 Arc	12.61	x	17.889	23.387
7 Arc	19.084	x	23.747	32.864
8 Arc	13.957	24.704	19.993	22.499
11 Arc	13.134	24.738	x	23.236
16 Arc	13.709	x	x	x
17 Arc	13.84	24.952	20.393	x
Mean	14.389	24.798	20.5055	25.4965
STDEV	2.3551355	0.13444702	2.42409481	4.92696367
SEM	0.96148	0.07762302	1.21204741	2.46348183
Median	13.7745	24.738	20.193	23.3115
Range	6.474	0.248	5.858	10.365

Group B: Clomiphene Citrate

	B-actin	Kisspeptin	Dynorphin	Neurokinin
2 AV	11.434	28.749	16.944	1.994
5 AV	13.464	24.681	20.59	21.334
6 AV	18.028	24.498	20.298	47.007
13 AV	12.752	26.282	17.716	22.386
14 AV	14.569	29.999	18.764	24.685
15 AV	12.262	26.889	18.072	22.456
Mean	13.7515	26.84966667	18.73066667	23.3103333
STDEV	2.350652314	2.194675891	1.453983035	14.3059285
SEM	0.959649789	0.895972681	0.593586088	5.84037087
Median	13.108	26.5855	18.418	22.421
Range	6.594	5.501	3.646	45.013

	B-actin	Kisspeptin	Dynorphin	Neurokinin
2 Arc	x	23.544	18.616	20.019
5 Arc	13.352	25.024	17.667	21.918
6 Arc	13.635	26.394	19.307	19.774
13 Arc	12.664	27.334	19.346	20.246
14 Arc	12.967	24.043	19.719	20.664
15 Arc	x	24.861	20.619	22.093
Mean	13.1545	25.2	19.2123333	20.7856667
STDEV	0.4264712	1.42873888	1.00059176	0.99081677
SEM	0.2132356	0.58328021	0.40848987	0.40449925
Median	13.1595	24.9425	19.3265	20.455
Range	0.971	3.79	2.952	2.319

Group C: Clomiphene Citrate + KpA

	B-actin	Kisspeptin	Dynorphin	Neurokinin
1 AV	12.943	22.406	18.984	22.328
4 AV	xx	xx	xx	xx
9 AV	12.869	30.319	17.77	22.926
10 AV	12.061	28.801	19.506	22.11
12 AV	x	x	27.437	46.289
18 AV	13.796	32.262	19.229	24.028
Mean	12.91725	28.447	20.5852	27.5362
STDEV	0.70907375	4.269176579	3.887168211	10.5094947
SEM	0.354536875	2.13458829	1.738394472	4.6999889
Median	12.906	29.56	19.229	22.926
Range	1.735	9.856	9.667	24.179

	B-actin	Kisspeptin	Dynorphin	Neurokinin
1 Arc	12.997	24.396	18.491	x
4 Arc	13.827	24.795	20.126	23.341
9 Arc	x	24.363	18.463	x
10 Arc	12.987	24.805	20.35	x
12 Arc	x	25.555	21.813	x
18 Arc	x	24.783	19.651	22.192
Mean	13.270333	24.7828333	19.8156667	22.7665
STDEV	0.4821134	0.42960001	1.26354449	0.81246569
SEM	0.2783483	0.17538347	0.51583988	0.5745
Median	12.997	24.789	19.8885	22.7665
Range	0.84	1.192	3.35	1.149

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APPENDIX B - Statistical and Imputation Analysis

Av_Act						P-value Post-Imputation (Pre-Imputation)		
						[95% confidence Interval]		
	Observed N	**Observed Mean (sdev)**	**Imputed N**	**Imputed Mean**	**Sdev**	{95% confidence interval rewritten}		
Group A	5	13.391 (0.857)	6	13.287	0.807	A vs B	A vs C	B vs C
Group B	5	12.896 (1.192)	6	12.973	1.082	0.497 (0.473)	0.411 (0.405)	0.932 (0.976)
Group C	4	12.917 (0.709)	6	12.558	0.799	[-1.926 ; 1.044]	[-1.975 ; 0.935]	[-1.711 ; 1.589]
						{0.86 ≤ μB/μA ≤ 1.08}	{0.85 ≤ μC/μA ≤ 1.07}	{0.87 ≤ μC/μB ≤ 1.12}

Arc_Act						P-value Post-Imputation (Pre-Imputation)		
						[95% confidence Interval]		
	Observed N	**Observed Mean (sdev)**	**Imputed N**	**Imputed Mean**	**Sdev**	{95% confidence interval rewritten}		
Group A	5	13.450 (0.566)	6	13.541	0.553	A vs B	A vs C	B vs C
Group B	4	13.155 (0.426)	6	13.184	0.56	**0.578 (0.4171)**	**0.677 (0.664)**	**0.941 (0.750)**
Group C	3	13.270 (0.482)	6	13.552	0.597	[-1.031 ; 0.633]	[-1.152 ; 0.815]	[-1.025 ; 1.085]
						{0.92 ≤ μB/μA ≤ 1.05}	{0.91 ≤ μC/μA ≤ 1.06}	{0.92 ≤ μC/μB ≤ 1.08 }

Av_Kiss						P-value Post-Imputation (Pre-Imputation)		
						[95% confidence Interval]		
	Observed N	**Observed Mean (sdev)**	**Imputed N**	**Imputed Mean**	**Sdev**	{95% confidence interval rewritten}		
Group A	5	24.069 (2.427)	6	23.471	2.619	A vs B	A vs C	B vs C
Group B	6	26.850 (2.195)	6	26.85	2.195	**0.082 (0.077)**	**0.061 (0.092)**	**0.405 (0.453)**
Group C	4	28.447 (4.269)	6	28.906	3.686	[-0.472 ; 6.33]	[-0.276 ; 9.61]	[-2.917 ; 6.397]
						{0.98 ≤ μB/μA ≤ 1.26}	{0.99≤ μC/μA ≤ 1.41}	{0.89 ≤ μC/μB ≤ 1.24}

Arc_Kiss						P-value Post-Imputation (Pre-Imputation)		
						[95% confidence Interval]		
	Observed N	**Observed Mean (sdev)**	**Imputed N**	**Imputed Mean**	**Sdev**	{95% confidence interval rewritten as ratio}		
Group A	3	24.798 (0.134)	6	24.689	0.705	A vs B	A vs C	B vs C
Group B	6	25.200 (1.429)	6	25.2	1.429	**0.642 (0.653)**	**0.928 (0.955)**	**0.512 (0.509)**
Group C	6	24.783 (0.430)	6	24.783	0.43	[-1.452 ; 2.187]	[-1.476 ; 1.377]	[-1.808 ; 0.974]
						{0.94 ≤ μB/μA ≤ 1.09}	{0.94 ≤ μC/μA ≤ 1.06}	{0.93 ≤ μC/μB ≤ 1.04}

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Av_Dyn						**P-value Post-Imputation (Pre-Imputation)**		
						[95% confidence Interval]		
	Observed N	**Observed Mean (sdev)**	**Imputed N**	**Imputed Mean**	**Sdev**	{95% confidence interval rewritten}		
Group A	6	20.419 (1.331)	6	20.419	1.331	A vs B	A vs C	B vs C
Group B	6	18.731 (1.454)	6	18.731	1.454	**0.0670 (0.0623)**	**0.108 (0.071)**	**0.883 (0.864)**
Group C	4	18.872 (0.765)	6	19.018	0.645	[-3.526 ; 0.150]	[-3.563 ; 0.453]	[-1.944 ; 2.210]
						{0.83 ≤ μB/μA ≤ 1.01}	{0.83 ≤ μC/μA ≤ 1.02}	{0.90 ≤ μC/μB ≤ 1.12}

Arc_Dyn						**P-value Post-Imputation (Pre-Imputation)**		
						[95% confidence Interval]		
	Observed N	**Observed Mean (sdev)**	**Imputed N**	**Imputed Mean**	**Sdev**	{95% confidence interval rewritten}		
Group A	4	20.506 (2.424)	6	20.235	1.929	A vs B	A vs C	B vs C
Group B	6	19.212 (1.001)	6	19.212	1.001	**0.384 (0.268)**	**0.695 (0.567)**	**0.385 (0.381)**
Group C	6	19.816 (1.264)	6	19.816	1.264	[-3.966 ; 1.756]	[-3.447 ; 2.444]	[-0.900 ; 2.106]
						{0.80 ≤ μB/μA ≤ 1.09}	{0.83 ≤ μC/μA ≤ 1.12}	{0.95 ≤ μC/μB ≤ 1.11}

Av_Neur						**P-value Post-Imputation (Pre-Imputation)**		
						[95% confidence Interval]		
	Observed N	**Observed Mean (sdev)**	**Imputed N**	**Imputed Mean**	**Sdev**	{95% confidence interval rewritten}		
Group A	6	22.609 (1.122)	6	22.609	1.122	A vs B	A vs C	B vs C
Group B	4	22.715 (1.410)	6	23.156	4.396	**0.882 (0.898)**	**0.285 (0.279)**	**0.376 (0.399)**
Group C	5	27.536 (10.509)	6	26.975	9.5	[-6.007 ; 6.746]	[-4.942 ; 14.630]	[-6.706 ; 15.655]
						{0.73 ≤ μB/μA ≤ 1.30}	{0.78 ≤ μC/μA ≤ 1.65}	{0.71 ≤ μC/μB ≤ 1.68}

Arc_Neur						**P-value Post-Imputation (Pre-Imputation)**		
						[95% confidence Interval]		
	Observed N	**Observed Mean (sdev)**	**Imputed N**	**Imputed Mean**	**Sdev**	{95% confidence interval rewritten}		
Group A	3	23.041 (0.475)	6	21.836	2.687	A vs B	A vs C	B vs C
Group B	6	20.786 (0.991)	6	20.786	0.991	**0.062 (0.008)**	**0.693 (0.656)**	**0.405 (0.405)**
Group C	2	22.766 (0.812)	6	21.115	2.666	[-3.824 ; 0.134]	[-5.121 ; 3.863]	[-2.622 ; 5.059]
						{0.82 ≤ μB/μA ≤ 1.01}	{0.77 ≤ μC/μA ≤ 1.18}	{0.87 ≤ μC/μB ≤ 1.24}

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