STUDY OF THE EFFECT OF ACETAMINOPHEN ON REPRODUCTION USING THE FEMALE MOUSE MODEL

A thesis submitted in fulfilment of requirements for a Doctor of Philosophy degree at University of Nairobi (Clinical studies -Theriogenology)

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DECLARATION

I hereby declare that this thesis is my original work and has not been presented for a degree at any other University.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

2-AG	2-arachidonoyl glycerol
ABHD-6/− 12	α, β-hydrolase-6 and -12
AEA	Anandamide
AEA	N-arachidonoyl ethanolamine
AIBP	AEA intracellular binding proteins
AKR1C1	Aldo-keto reductase
AR	Acrosome reaction
ARC	Arcuate
cAMP	Cyclic adenosine monophosphate
CB	Cannabinoids
CB1/2	Cannabinoid receptor 1/2
CL	Corpus luteum
CNS	Central nervous system
COX	Cyclooxygenase
COX-1	Cyclooxygenase-1
COX-2,	Cyclooxygenase-2
DAGL	Diacylglycerol lipase
ECS	Endocannabinoid system
EMT	Endocannabinoid membrane transporter

ER	Estrogen Receptor
E2	Oestrogen
FAAH	Fatty acid amide hydrolase
FABP	Fatty acid binding protein
FAO	Food Agriculture Organization
FSH	Follicle stimulating hormone
G-CSF	Granulocyte- Colony stimulating factor
GABA	Gamma-aminobutyric acid
GnRH	Releasing hormone
GnRH	Gonadotropin releasing hormone
GPCR	G-protein-coupled receptor
hCG	Human chorionic gonadotropin
HPO/G	Hypothalamic-pituitary-ovarian/gonadal
HSDs	Hydroxysteroid dehydrogenases
HSP	Heat shock protein
IFNτ	Interferon Tau
IL	Interleukin
IVF	In vitro fertilisation
KCSAP	Kenya Climate Smart Agriculture Productivity Project
LH	Luteinizing hormone

LOX	Lipoxygenase
MAG/MAGL	Monoacylglycerol / monoacylglycerol lipase
MAPK	Mitogen-activated protein kinase
NADA	N-arachidonoyl dopamine
NAPE	N-acylphosphatidylethanolamine
NAPE	N-arachidonoyl-phosphatidylethanolamine
NAPE-PLD	N-acylphosphatidylethanolamine-phospholipase D
NAPE-PLD	N-arachidonoyl-phosphatidylethanolamine phospholipase D
NSAID	Non-steroidal anti-inflammatory drugs
P4	Progesterone
PCOS	Polycystic ovarian syndrome
PG	Prostaglandin
PGE2	Prostaglandin E2
PGF2α	Prostaglandin F2α
PI3K-PKB	Phosphatidylinositol 3-kinase-protein kinase B
PLC	Phospholipase C
PPAR	Peroxisome proliferator-activated receptors
THC	Δ9-tetrahydrocannabinol
TNF	Tumour necrosis Factor
TRAIL	Tumour necrosis factor related a

TRPV1	Transient receptor potential vanilloid type 1
VGEF	Vascular Endothelial Growth Factor
ZP	Zona pellucida

ABSTRACT

Acetaminophen is not considered a typical non-steroidal anti-inflammatory drug (NSAID) and its effects on reproduction have not been studied extensively as other NSAIDs. Acetaminophen is known to act through the inhibition of prostaglandin synthesis and activation of the endocannabinoid system, thus can be associated with the regulation of mammalian reproductive processes through the same systems. To provide further evidence, this study was devised to establish the effect of acetaminophen on female reproduction. Specifically, its impact on the oestrous cycle, reproductive hormone production, ovulation, and implantation.

Female Swiss white mice aged between six and eight weeks were used for the study. The mice were sourced from Kenya Medical Research Institute (KEMRI) and International Livestock Research Institute (ILRI). The mice were randomly divided into control and treatment groups of five (5) mice each. Throughout the experiments, mice in the treatment group received 200mg/kg of acetaminophen, while the control group received 0.5ml of normal saline via oral gavage.

There was no significant difference in the number and length of oestrous cycles was observed in the treatment (3.54±0.155 and 5.52±0.187) and control (3.54±0.144, and 5.38±0.172) groups from the study. There was however, a significant reduction in the frequency of proestrus in the treatment group ($P \leq 0.05$). There was no significant difference in FSH, LH, and estrogen levels. However, there was a delay in the production of the FSH, LH, and estrogen, with peak production seen during metestrus. There was a significant reduction in progesterone levels in the treatment group ($P \leq 0.05$). A significant reduction in the number of implantation sites in mice treated with acetaminophen in pre ($P \leq 0.05$) and post-coitus ($P \leq 0.01$) treatment regimes was also seen. Upon withdrawal of acetaminophen, implantation sites of mice in the treatment group did not differ from those in the control group.

On histology there was an increase in the number of secondary follicles in the treatment group compared to the control. Both secondary and tertiary follicles had degenerative changes in the treatment group. There was also a disruption on the endometrial lumen and a reduction in the number of villi in the treatmetn group when compared to the control.

From the study, acetaminophen demonstrated some negative effects on reproduction in the mice and, therefore, has potential for use to lower fertility. However, further studies are required to fully study how it affects reproduction in females.

CHAPTER 1

1.0 BACKGROUND INFORMATION

Acetaminophen, commonly known as Paracetamol, is an over-the-counter medication found either as a single molecule or in combination with other drugs. Despite its use as an analgesic for about a century, the precise mode of action is still not completely understood. Acetaminophen is known to inhibit prostaglandin production, and in the reproductive system, prostaglandins are essential in the formation and degeneration (lysis) of the corpus luteum . Prostaglandin E2 is luteotrophic, while Prostaglandin F2α is luteolytic in nature. Inhibition of their production may result in either failure to develop or prolong the lifespan of the corpus luteum, respectively. A study by Albert et al., (2013) shows that Acetaminophen has disruptive endocrine properties in adult human male testis In vitro. Consequently, this study evaluated the effect of acetaminophen on female reproduction using the female mouse.

In recent years, Acetaminophen has been shown to exert analgesia through action on the endocannabinoid system. Specifically, through its conversion to N-arachidonoylphenolamine (AM404), Acetaminophen stimulates the endocannabinoid system by inhibiting the reuptake of anandamide (Högestätt et al., 2005). The endocannabinoid system (ECS) is involved substantially in controlling reproductive events and the synchronous growth of the embryo and the endometrium to facilitate implantation. In light of this, this study sought to answer the question: 'Does Acetaminophen interfere with implantation?'

Anandamide (AEA) has been implicated in miscarriages, with studies showing plasma anandamide levels exceeding 2 nM led to miscarriage while plasma levels of <2 nM resulted in live births. Therefore, the preceding indicates that modifying the levels of Anandamide is a potentially viable approach to developing novel, efficient and safe contraceptives. More importantly, it also shows that a change in this system can have adverse effects on reproduction and, therefore, potential use as a contraceptive and hence the use of Acetaminophen.

This study aimed to investigate the effect of Acetaminophen on female reproduction and its potential as a contraceptive by determining its impact on the oestrous cycle, reproductive hormone levels, implantation, and histomorphology of the ovary and uterus in the female mouse.

1.1 OBJECTIVES

1.1.1 GENERAL OBJECTIVE

To determine the effect(s) of acetaminophen on female reproduction.

1.1.2 SPECIFIC OBJECTIVES

1. To evaluate the anti-fertility effect of acetaminophen on the oestrous cycle of the female mouse.
2. To determine the anti-fertility effects of acetaminophen on implantation in the female mouse.
3. To examine the anti-fertility effect of acetaminophen on the number and quality of corpora lutea and histomorphological changes of the ovary and endometrium in the female mouse.

1.2 PROBLEM STATEMENT

A stray dog is defined as any dog in a public area that is not under direct human control (Gill et al., 2022). This term therefore incorporates both unowned and community-owned dogs. Stray dogs may be previously owned dogs or entirely feral dogs (wild dogs) (Miklosi, 2007; Mota-Rojas et al., 2021; Shimozako et al., 2018).

The actual stray dog population contributes a small percentage of the roaming dog population. The current spay/neuter release approach for unowned dogs only serves to reduce this particular population but does not solve the stray dog menace experienced globally. Dealing with the stray dog population amounts to managing symptoms instead of getting to the root cause. Identifying the source of the dogs on the streets should be the focus of dog population management.

The current assumption is that the rising dog population is from breeding unowned dogs. While these animals are thought to reproduce prolifically, however, the survival rate of their offspring

is low compared to the owned dogs. The recruitment process is also low as very few of the puppies born reach adulthood. Most free-roaming dogs were previously owned dogs that were either abandoned, left to roam unsupervised, or got lost accidentally. Owned dogs are likely to have better access to food and other resources, improving their puppies' survival rate and increasing the recruitment rate to the adult pool. There are four different sub-populations of dogs in communities; owned, lost, community, and unowned. Therefore, these should all be targeted for population control.

Most breeders might not agree to sterilise their animals due to loss of income. There is a need to manage the number of puppies born for commercial purposes. This can be done by managing the litter sizes for both owned and unowned dogs in the street. A contraceptive/drug that can achieve this may be better than permanently sterilising a female and male animal. The uptake may also be better than permanent sterilisation since dogs only come into heat twice a year. It will also reduce the risk of general anaesthesia associated with any surgical procedure and the ensuing undesired weight gain. Puppy milling has also become a source of the unowned dog population. There is a high likelihood that the parent stock is kept in suboptimal conditions contravening the five freedoms to which animals are entitled. Puppies born in this condition are usually weak and susceptible to diseases increasing the risk of abandonment.

Several contraceptives are available for human consumption in the market, and several others have been suggested for animal use. However, a majority of these agents interfere with the female endocrine system. This interference has been associated with multiple side effects, including excessive menstrual bleeding, irregular cycles, and irreversible fertility issues leading to hospitalisation. In animals, contraceptives are associated with weight gain and an increase in the occurrence of pyometra. The discovery of a non-hormonal contraceptive will help in waylaying the fears associated with using contraceptives and hence improve uptake

while achieving the desired population control. Acetaminophen will also provide a cheaper and readily available alternative to population control.

1.3 JUSTIFICATION

There has been a need to develop a contraceptive devoid of hormonal interference, particularly in humans and small animals. Many alternatives have been tried, including a discussion on using acetaminophen. There has been a large population of women and small animals reporting side effects following the use of hormonal contraceptives. Acetaminophen is one of the options that can be used as an alternative to hormonal contraceptives.

Current hormonal contraceptives in animals are expensive and not readily available, while in humans, they are associated with physiological side effects (Ochako et al., 2015). Acetaminophen is a commonly used analgesic in animals and humans (Babb et al., 2010) and is suspected of having a contraceptive ability. In the best embodiment, acetaminophen inhibits the re-uptake of anandamide through its conversion to AM404, increasing extracellular levels of anandamide. This stimulates the endocannabinoid system resulting in asynchronous development of the embryo and endometrium, leading to implantation failure. Or, through its action on cyclooxygenase, acetaminophen will inhibit prostaglandins production, either luteotropic- PGE or luteolytic -$PGF2_{\alpha}$, affecting the quality of corpus luteum and, in turn, impairing the production of progesterone leading to pregnancy failure. It is thus worthy of elucidating such suspicion through scientific research and if proven for use in the future as an alternative contraceptive to those based on hormones.

The study used the mouse animal model because the mouse uterus has been shown to have high levels of anandamide (Schmid et al., 1997), and their short oestrous cycle makes them ideal for the study. The design followed the recommended animal welfare guidelines. It used a pre-tested non-detrimental oral dosage rate of 200mg/kg, which is lower than the

recommended dosage of 300mg/kg (Hawk et al., 2005). If scientifically proven, the clinical application of acetaminophen as a contraceptive would be a patentable breakthrough as a nonhormonal contraceptive in both animals and humans. It will also safeguard against a fundamental hormonal contraceptive side-effect challenge experienced in reproduction (Wang et al., 2006). Indeed, it will be cheaper and widely used to control the stray dog population menace experienced worldwide in developing countries (Mutembei et al., 2015). Therefore, it is essential to undertake research and explore the anti-fertility properties of acetaminophen to offer affordable alternative forms of contraceptive technologies.

CHAPTER 2

LITERATURE REVIEW

2.0 INTRODUCTION

Over the last four decades, researchers have explored non-surgical methods to suppress animal fertility (Rhodes, 2017). Several ways have been reported over the years, including; Immuno-contraception in wildlife and deslorelin implant in male dogs. These methods, however, do not confer long-term immunity and are not available locally.

Similarly, in 2012, a lack of modern contraceptives affected over two hundred million women in developing countries, resulting in approximately fifty-four (54) million unintended pregnancies and over seventy thousand maternal deaths (Darroch and United Nations, Department of Economic and Social Affairs, 2015). In Kenya, there is a preference for short-acting instead of long-acting contraceptives (Izugbara et al., 2018). One in three users discontinues use within the first year of intake mainly due to side effects. There is still an unmet need for contraceptives in the market, especially modern ones. Developing a short-acting nonhormonal contraceptive can help meet this demand without the side effects of hormonal contraceptives.

2.1 ANATOMY OF THE FEMALE REPRODUCTIVE SYSTEM

The female mouse reproductive system includes the ovaries, oviducts, uterus, vagina, mammary glands, and the placenta and foetus in pregnant animals (Figure 1).

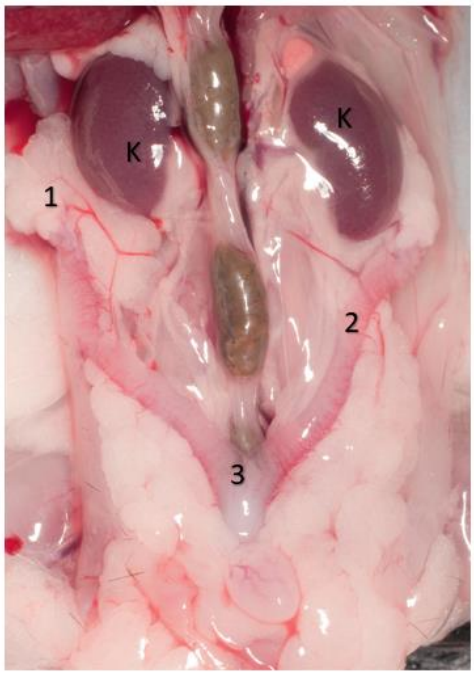

Figure 1: Gross anatomy of the mouse reproductive system in situ.

Paired ovaries (1) are located at the caudal poles of the kidneys (K) embedded in the ovarian fat pad. Paired uterine horns (2) meet at the fundus (3).

The ovaries in mice are spherical and located at the caudal poles of the kidneys. The mesovarium holds them in place, which attaches to the dorsal body wall. The ovarian bursa covers the entire ovary except for the hilus, where blood vessels and nerves pass through. A single cuboidal to columnar epithelial cell layer makes up the germinal epithelium. The parenchyma comprises a compact outer layer – the cortex, and an inner layer – the medulla. The cortex has developing follicles, corpora lutea, blood vessels, and supporting stroma, while the medulla comprises connective tissues and blood vessels.

The mouse oviduct is a narrow, coiled tube about 1.8cm long connecting the uterine horn with the periovarian space. It is suspended from the dorsal body wall by the mesotubarium. The oviduct has a fimbriated end towards the ovary and an intramuscular layer connecting it to the uterus. The body is divided into the infundibulum, ampulla, and isthmus from the ovary towards the uterus. The mucosa of the ampulla and infundibulum is composed of a tall,

columnar epithelium with prominent long, motile cilia. The isthmus is composed of low pseudostratified columnar cells with variable cilia numbers. In mice, the intramuscular segment extends into the dorsolateral uterine wall forming the colliculus tubarius.

Mice have a bicornuate uterus suspended by the mesometria through which blood vessels, lymphatics, and nerves are supplied. The uterine wall has three layers; the endometrium, myometrium, and serosal layer. The endometrium consists of simple columnar cells and loosely arranged reticular connective tissue. The myometrium consists of smooth muscle fibres arranged in an inner circular layer and an outer longitudinal layer separated by loose, highly vascular connective tissue, the stratum vasculum. The outer serosa is continuous with the mesometrium.

2.2 OESTROUS CYCLE AND ENDOCRINE CONTROL OF REPRODUCTION

The oestrous cycle denotes the cyclic pattern of ovarian activity that enables a female animal to move from a period of reproductive receptivity to non-receptivity with the ultimate goal of establishing a pregnancy after mating (Crowe, 2020). There are two phases of the cycle: follicular and luteal phases.

The follicular phase involves the recruitment, selection, and maturation of follicles and ends at ovulation. The luteal phase consists of the formation of the corpus luteum by luteinisation of granulosa and theca cells. Once a mature tertiary follicle undergoes ovulation, it is converted into a corpus haemorrhagicum, then to the progesterone-producing corpus luteum.

Hormones produced by the hypothalamus, pituitary gland, and ovary (hypothalamo-pituitary-gonadal axis- HPG) regulate the oestrous cycle in mammals. In summary, Gonadotropin Releasing hormone (GnRH) is released from the hypothalamus into the hypophyseal-portal system; from here, it travels to the anterior pituitary, where it triggers gonadotrophins release luteinising hormone (LH) and follicle-stimulating hormone (FSH) into the circulation. These are then transported to the testes and ovaries, where they stimulate the production and release

of sex steroid hormones, testosterone, oestrogen (E2), and progesterone. These sex hormones are then transported back to the hypothalamus through the circulation to provide positive or negative feedback, completing the HPG axis (McIlwraith and Belsham, 2020).

2.2.1 HYPOTHALAMUS

The control of reproductive function is an intricate process requiring the synchronised effort of several well-defined neurons in the hypothalamus (Schauer et al., 2015). Gonadotropin-releasing hormone neurons located in the hypothalamus are the critical regulators of puberty and fertility. GnRH neurons are primarily found in the medial preoptic area and adjacent to the median eminence(Herde *et al.,* 2013). The median eminence is a fenestrated blood-brain barrier area located at the hypothalamus's base. The porous nature of the median eminence enables it to sense and respond to chemical signals carried by the circulatory system within the circumventricular organs in the brain. This allows 'cross-talk'/signalling between the hypothalamus and periphery (Yin & Gore, 2010). These neuropeptide-expressing neurons obtain signals from both afferent neurons in the central nervous system and peripheral cues on the physiological status of the animal and whether the circumstances are conducive for reproduction (McIlwraith and Belsham, 2020). Some of the peripheral cues include hormones (oestrogen, insulin, and leptin), nutritional components (fatty acids and proteins), and inflammation signals (McIlwraith and Belsham, 2020).

GnRH neurons receive input from afferent neurons and act synergistically to coordinate reproduction. Kisspeptin (Kiss1) is an essential regulator of GnRH neurons. Kisspeptin was first discovered in 2001 in the human placenta as an endogenous ligand of the G protein-coupled receptor gene, GPR54 (Ohtaki et al., 2001). Recent evidence has shown that kisspeptin neurons in the hypothalamus play an essential role in controlling the onset of puberty by triggering GnRH release. Hence, kisspeptin neurons act as the GnRH pulse generator. Kiss1 is expressed in the hypothalamus's arcuate (ARC) and anteroventral periventricular (AVPV)

nuclei. The ARC Kisspeptin neuron population regulates pulsatile tonic GnRH production, whereas the AVPV Kiss1 neuron population controls the female pre-ovulatory LH surge (Hu et al., 2015; McIlwraith and Belsham, 2020). These include proopiomelanocortin (POMC) and neuropeptide Y. Other neurons in the hypothalamus coordinate energy homeostasis (NPY). In addition, these neurons (NPY and POMC) communicate with reproductive neurons to indicate nutritional status. In addition to being a crucial regulator of pulsatile GnRH production, ovarian oestrogen is also a crucial regulator of GnRH production. During the preovulatory oestrogen surge, it produces positive and negative feedback on GnRH neurons. Hypogonadotropic hypogonadism has been linked to an absence of oestrogen signaling.

From the hypothalamus, GnRH is released into the hypophyseal-portal system. From here, it travels to the anterior pituitary gland. Pulsatile secretion of Gonadotropin-releasing hormone (GnRH) from the hypothalamus facilitates the neuroendocrine control of reproduction in both females and males.

2.2.2 PITUITARY GLAND

In response to the pulsatile production of GnRH, the anterior pituitary gland secretes the gonadotropins, Follicle-stimulating hormone (FSH), and luteinising hormone (LH), which ultimately control gonadal function. FSH stimulates follicles in the ovary to grow and mature; LH stimulates ovulation and corpus luteum formation.

2.2.3 OVARY

The ovary's primary role is to produce oocytes for fertilisation and oestrogen and progesterone. The follicle is the functional unit of the ovary (Hannon & Curry, 2018; Eppig, 2002). It contains the developing oocyte, surrounding granulosa, and theca cells (Hannon and Curry, 2018). Folliculogenesis is the process whereby the follicle undergoes some unalterable developmental changes from a primordial follicle to the ovulatory antral follicle (Hannon & Curry, 2018).

Folliculogenesis is complex and dynamic, involving several ovarian and endocrine cells and numerous signals (Hannon & Curry, 2018).

Primordial germ cells migrate from the yolk sac to the genital ridge during embryological development, where the undifferentiated gonads are found (Hannon & Curry, 2018). The germ cells (oogonia) undergo mitotic division to reach peak species-specific numbers. About 6-7 million oogonia assemble to establish the germ cell nest in humans. Once the nest is established, mitotic division ceases, and the now oocytes undergo meiotic division. The oocytes undergo meiosis up to the diplotene stage of prophase 1. 'Primordial follicle assembly occurs via germ cell nest breakdown' (Hannon and Curry, 2018). This process involves some oocytes' atresia found within the germ cell nests. The interaction of several molecular events that leads to oocyte death causes the remaining oocytes to become associated with a single layer of flattened, squamous granulosa cells, resulting in the formation of the primordial follicle (Hannon & Curry, 2018).

Primordial follicles are the sole source of germ cells throughout the entirety of a woman's reproductive life. They are continuously recruited in cohorts to initiate folliculogenesis after activation. This activation requires about two weeks in mice and up to six months in humans (Kaingu et al., 2016; Sánchez & Smitz, 2012). Primordial follicle activation is a very complex and precise process, and despite significant progress, many molecular mechanisms remain poorly understood (Kaingu et al., 2016). Primordial follicles are the first-class follicles that develop in the ovaries of mammals and consist of an oocyte surrounded by a solitary pre-granulosa squamous cell. There are four possible outcomes for each primordial follicle: (I) quiescence, (ii) attrition, (iii) initiation of development followed by atresia, and (iv) ovulation followed by the formation of a short-lived corpus luteum.

Oogonia, which originates from primordial germ cells, multiply by mitosis and form primary oocytes that arrest at the prophase stage of the first meiotic division until they are fully grown. The default fate of the primordial follicle is to remain quiescent and immature. However, the rate-limiting transition of preliminary recruitment into a primary follicle is species-specific and can occur at any stage of life (Hannon and Curry, 2018). This transition is gonadotropin independent since primordial follicles lack gonadotropin receptors and are devoid of theca cells. The changes, therefore, rely on intraovarian factors and signals. There is a delicate balance between stimulatory and inhibitory factors involved in the transition, with stimulatory factors enhancing the growth and development of the primordial follicle.

In contrast, inhibitory factors discourage growth and enhance the survival of the primordial follicles. Some of the stimulatory factors studied so far include kit ligand, basic fibroblast growth factor (bFGF), keratinocyte growth factor (KGF), leukaemia inhibitory factor (LIF), bone morphogenetic proteins 4 and 7 (BMP4 and BMP7) . Platelet-derived growth factor (PDGF) showed an intense increase in preliminary recruitment, as evident by a decrease in primordial follicle numbers and an increase in growing follicle numbers. Anti-Müllerian hormone (AMH), which belongs to the transforming growth factor-beta (TGF-b) superfamily, inhibits initial recruitment. Although the exact mechanism by which anti-müllerian hormone subdues primary recruitment remains undetermined (Hannon and Curry, 2018).

During the transition to the primary follicle, the granulosa cells surrounding the primordial cell change in shape from squamous to cuboidal. The oocyte increases in size and is surrounded by the zona pellucida. Primary follicles are small, surrounded by a single layer of cuboidal granulosa cells within an outer basal lamina (Eppig, 2002). The zona pellucida surrounds the oocyte. Mechanisms that control the start of follicular growth (follicle activation) and the subsequent growth and differentiation of preantral follicles are of great interest (Eppig, 2002).

Their explanation is essential to using the primordial pool to improve reproductive efficacy in domestic animals, humans, and endangered species (Eppig, 2002).

Secondary follicles are larger than primary follicles (Figure 2). They consist of the primary oocyte with its zona pellucida surrounded by several layers of granulosa cells, a basal lamina, and a theca layer. The theca layer forms when there are two or three granulosa cell layers. Theca cells are recruited from surrounding interstitial stroma (Baird and Mitchell, 2002). The transition from a primary to a preantral/tertiary follicle is mainly driven by intraovarian factors and signalling pathways (Hannon and Curry, 2018).

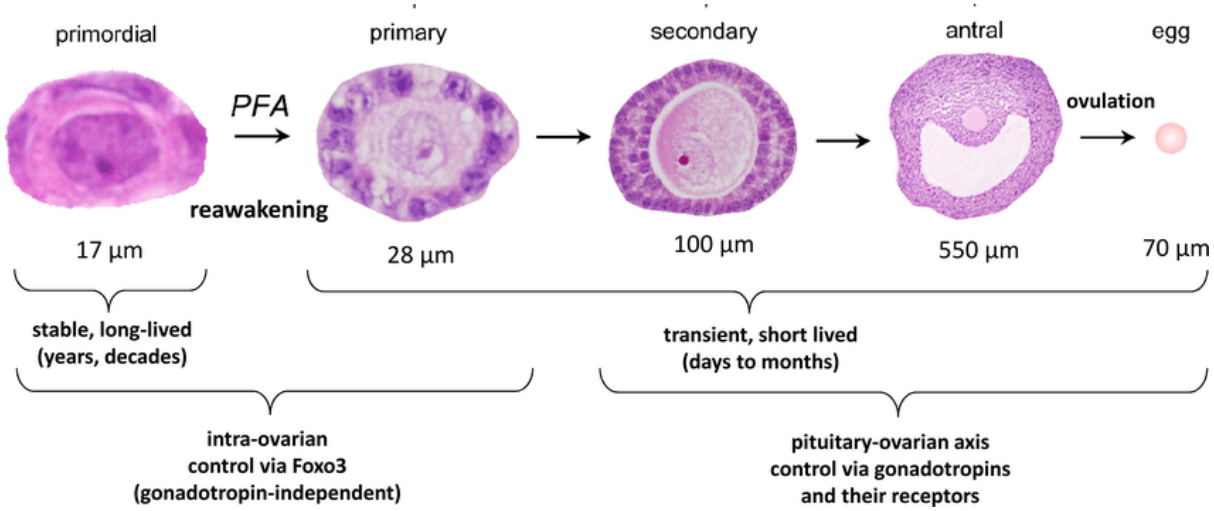

Figure 2: Stages of follicular maturation in Mice.

The presence of the antrum signals the formation of the tertiary follicle. The oocyte is surrounded by cumulus cells derived from the granulosa cells. Even though the mitotic rate of the granulosa and theca cells begins to fall in tertiary follicles, the antral fluid content increases and follicles continue to grow in size. The granulosa layer undergoes functional modifications that allow the follicle to release an oocyte at ovulation and luteinise somatic cells in response to the preovulatory gonadotropin surge.

Before ovulation, the number of viable large antral follicles is reduced to a species-specific number. Only one ovulatory follicle emerges in monotocus species such as humans and

ruminants, while the rest undergo atresia. This process of selection is not fully understood. It, however, involves three crucial steps: (a) secondary recruitment, (b) selection, and (c) dominance (Baird and Mitchell, 2002; Smitz and Cortvrindt, 2002). Secondary recruitment occurs from the pool of healthy small antral follicles (2-4 mm diameter) present in the ovary during luteal regression.

As the levels of oestradiol, progesterone, and inhibin-A fall, the secretion of FSH and LH increases. The concentration of FSH rises above that required to activate the mechanisms necessary for the final differentiation of the follicles, including stimulation of aromatase and LH receptors in the granulosa cells. This maturational process is reflected by a rise in the peripheral concentration of inhibin B, which occurs within 48 h of the increase in FSH (Baird and Mitchell, 2002). mRNA for activin/inhibin /3~ subunit is present in granulosa cells of even preantral follicles (McNatty et al., 2000). Although of similar size, one follicle is presumed to be slightly more developed, so it can respond immediately to the rise in FSH. Several intraovarian factors are known to alter the responsiveness of the granulosa and theca cells to FSH and LH. For example, activin and insulin-like growth factor (IGF) I and II enhance the ability of FSH to induce aromatase (Campbell, 1999; Czieselsky et al., 2016).

Thus the "chosen" follicle can benefit maximally from the rise in FSH and develop faster than other minor follicles. Once selected, the dominant follicle must suppress the further development of other antral follicles, which have the potential to ovulate. The mechanism involves suppressing FSH below the threshold necessary to activate and support the growth and maturity of other follicles. During the mid to late follicular phase, there is a distinct rise in the concentration of inhibin A and oestradiol originating almost entirely from the dominant follicle (Groome et al., 1996). In this way, the ovulatory follicle has total control over the feedback signals, which control the secretion of gonadotrophins by the anterior pituitary. Two possible mechanisms have been suggested through which the dominant follicle sustains its

growth and development during the final stages: The granulosa cells of the dominant follicle acquire significant numbers of LH receptors around the time of selection (Webb and England, 1982). the levels of LH in the follicular phase of the cycle rise in association with a marked increase in the frequency of LH pulses in contrast to those of FSH (Baird and Mitchell, 2002). This rise in LH stimulates androgen production from the theca cells, guaranteeing an ample supply of precursors for oestradiol synthesis. In addition, as the granulosa cells acquire LH receptors, they can progressively utilise LH and FSH via cyclic adenosine monophosphate (cAMP) production. In this way, the late follicular phase LH may be used as a partial substitute for FSH to promote the final stage of folliculogenesis (Baird and Mitchell, 2002).

It is also likely that local autocrine and paracrine factors increase the sensitivity of the dominant follicle to FSH. For instance, insulin-like growth factor (IGF) enhances the responsiveness of granulosa cells to FSH in vitro (Robertson et al., 2015). In small follicles, the bulk of IGF is inactivated by binding to insulin-like growth factor binding protein (IGFBP). In some species, there is a high concentration of unbound IGF in the preovulatory follicle due to the selective degradation of IGFBP (Campbell and Baird, 2001; Fortune, 2003; Mazerbourg et al., 2000). It is likely that inhibin and oestradiol act locally within the follicles and act as critical endocrine signals. In several species, it has been demonstrated that inhibin enhances LH-stimulated androgen production by theca (Hillier et al., 1991), and FSH stimulated aromatase activity in sheep granulosa cells (Campbell and Baird, 2001). In this way, the dominant follicle becomes increasingly sensitive to gonadotrophins and can survive in an environment hostile to subordinate follicles' recruitment and further development (Baird and Mitchell, 2002).

Following ovulation, the remainder of the follicle wall forms a corpus luteum. Granulosa cells differentiate into large luteal cells (LLC), while the theca cells form small luteal cells. The LLC makes up 30% of the steroidogenic cells and secretes 70% of the progesterone, while the SLC comprises 70% of the steroidogenic cells but secretes only 30% of the progesterone (Weems

et al., 2004). SLC requires LH stimulation for maximal progesterone secretion. In the absence of pregnancy, the endometrium produces PGF2α, which is transported to the ovary for luteolysis. PGF2α reaches the CL via local and systemic transport, depending on the species. Specifically, PGF2α is secreted into the uterine vein and is transferred locally to the ovarian artery of the luteal containing ovary in the cow, ewe, and sow; however, in mares, it is transported via systemic circulation since little contact occurs between the uterine vein and ovarian artery (Ginther et al., 2011). The corpus luteum is lysed to initiate a follicular wave. Luteal regression proceeds in two steps: First, there is the decrease in P4, which is considered functional luteolysis, and then luteal involution is described as structural luteolysis (Arosh et al., 2004).

Endocrine-disrupting chemicals can act on these pathways and disrupt reproductive function.

Follicular atresia and luteal regressions are essential mechanisms required to eliminate unnecessary and aged structures and normal ovarian function (Bukovsky et al., 2006). The development of small preantral follicles is gonadotropin-independent. Therefore, an increase in atresia in these follicles is usually seen with direct-acting cytotoxic compounds, heavy metals, or radiation (Vidal and Dixon, 2018).

2.2.4 VAGINAL CYTOLOGICAL CHANGES IN THE OESTROUS CYCLE

The mouse is polyoestrous and cycles every 4-5 days unless interrupted by pregnancy or pseudopregnancy. The oestrous cycle in mice has four phases: proestrus, oestrus, metestrus, and dioestrus. Vaginal cytology is used to identify the phases of the oestrous cycle and is also an indicator of the functional status of the reproductive system (Cora *et al.*, 2015). Vaginal cytology identifies oestrous stages by the presence/absence, percentage, concentration, and distribution of nucleated epithelial cells, cornified epithelial cells, and leukocytes on a slide (Cora *et al.*, 2015).

Proestrus is short and lasts less than 24 hours (Cora *et al.*, 2015; Grasso *et al.*, 1998). According to Cora *et al*. (2015), "this stage is characterised by the occurrence of small, round, nucleated epithelial cells of relatively uniform size and appearance; these cells may stain deeply basophilic and organised in grape-like clusters, sheets, or strands, with few to no neutrophils seen". These sheets, clusters, and strands may not be observed in low cellularity samples. The uterus in proestrus is distended and hyperaemic with mitotic cells observed in the epithelial cells, and leukocytes infiltrate the stroma.

Oestrus ranges between 12 and 48 hours (Cora et al., 2015; Grasso et al., 1998) with mainly anucleated keratinised epithelial cells with numerous bacteria occasionally observed free or adhered to the cells in the background (Cora et al., 2015). Mitosis is peaking in the uterus during oestrus, and leukocytes are rare. Metestrus lasts for about 6-8 hours with the presence of anucleated keratinised epithelial cells. The uterine wall is flaccid in metestrus, degenerative endometrial cells with no mitoses, and prominent leukocytes.

Dioestrus is the longest stage in the mice's oestrous cycle lasting 48-72 hours with a substantial decrease in anucleated keratinised epithelial (Cora *et al.*, 2015). In dioestrus, the uterine wall collapses, and leukocyte infiltration and reformative changes are observed in the endometrium. The uterine mucosa is absorbed during dioestrus. Although not as striking as the changes seen in the mouse vaginal epithelium, the morphology of the mouse endometrium changes depending on the oestrus cycle

The mouse oestrous cycle is short, with ovulation occurring every 4 –5 days (Miller and Takahashi, 2014). A luteinising hormone surge precedes ovulation in response to the positive feedback effect of oestrogen produced by follicular cells, and in mice, this occurs in the late afternoon of proestrus (Fride, 2002; Miller and Takahashi, 2014). Ovulation occurs 12–14 hours after the onset of proestrus (Miller and Takahashi, 2014).

2.3 FERTILISATION AND EMBRYOGENESIS

Fertilisation is the union between a viable male and female gamete to form a diploid organism, a zygote. Fertilisation takes place within the ampulla of the oviduct. As a prerequisite, the spermatozoa and the ovum have to travel from the uterus and ovary, respectively to the oviduct.

2.3.1 OVUM TRANSPORT

There are two steps in ovum transport. The first step is oocyte pick-up from the surface of the ovary or the ovarian bursa by the fimbriae (Suarez, 2015). The second is the oocyte's transport to the oviduct's ampulla. Just before ovulation, the epithelial cells of the oviduct become highly ciliated, and smooth muscle activity within the tube and its suspensory ligament increase due to hormonal influences. At ovulation, the fimbriae of the oviduct move closer to the ovary and sweep rhythmically over its surface. In addition to the currents set up by the cilia, this action efficiently captures the ovulated oocyte/s (Yuan et al., 2021).

Once inside the oviduct, the ova are transported toward the uterus, mainly due to contractions of the smooth musculature of the oviduct. Even though the cilia lining the tubal mucosa as well play a role in egg transport, their action is not obligatory because humans with immotile cilia syndrome are often fertile.

Movement of the ovum along the oviduct usually takes 3–4 days, regardless of fertilisation. Egg transport normally occurs in two phases: first there is the slow transport in the ampulla which takes approximately 72 hours followed by a more rapid stage through the isthmus to the uterus which takes about 8 hours. The mechanism that temporarily inhibits the ovam from entering the isthmus of the oviduct is poorly understood. There is a possibility that local oedema or reduced muscular activity would prevent the ovum/embryo from entering the isthmus. Still, progesterone helps to relax the utero-tubal junction and allow the ovum/embryo to enter the isthmus. Sperm transport

During mating, the male deposits spermatozoa at various levels within the female genitalia: the anterior vagina in ruminants, rabbits, and humans and the uterus in sows. The hamsters and rats were also classified as uterine depositors because their sperm was found in the uterine horns soon after mating. However, Bedford and Yanagimachi, (1992) established that spermatozoa are deposited within the anterior vagina and then transported through the relaxed cervix into the uterus by muscular contractions of the female genital tract.

In murine rodents, following mating, most semen is quickly transported into the uterine cavity; however, some remains in the vagina, where it thickens to form a copulatory plug. This plug includes a cervical cap that enhances sperm transport into the uterus (Suarez, 2015). The plug extends into the cervical canal in guinea pigs and mice, preventing retrograde sperm loss. The copulatory plugs of rodents are mainly made of the seminal vesicle-secreted proteins (SVS) family. The mouse coagulating gland secretes transglutaminase, which polymerises SVS1-3 and is thought to be responsible for the formation of the plug.

The spermatozoa must traverse the cervix and uterus to reach the oviduct for fertilisation. While journeying through the uterus from where they are deposited, spermatozoa undergo capacitation and hyperactivation. The spermatozoa must undergo these two processes to attain fertilisation competence.

The first stage of the capacitation process is the removal of cholesterol from the sperm surface with the help of albumin and high-density lipoproteins found in the female genital tract (Suarez, 2015). Specifically, actin monomers are polymerised into F-actin polymers during sperm capacitation. The next stage of capacitation is removing the glycoproteins deposited on the sperm head within the epididymis. Capacitation is necessary for a spermatozoon to be able to fertilise an egg. Cholesterol and glycoprotein covering prevent premature capacitation (Serin et al., 2011).

Hyperactivation is marked by hypermotility of the spermatozoa allowing them to break free and efficiently make their way up to the ampulla (Suarez, 2015). Movements of spermatozoa within the female genitalia are facilitated by the peristaltic contraction of the myometrium and ciliary movement within the oviduct. Ultimately a few hundred spermatozoa reach the ova in the ampulla, and only one of them will fertilise an ovum.

On reaching the ampulla of the oviduct, the spermatozoa finding a viable ovum must penetrate the zona pellucida to achieve fertilisation. Contact between the head of the spermatozoa and the zona pellucida triggers an acrosome reaction. The acrosome reaction is an exocytotic process that enables the spermatozoon's passage through the zona pellucida and its subsequent fusion with the oocyte (Gupta and Bhandari, 2011).

During capacitation, the dispersion of actin polymers allows contact between the outer acrosomal membrane and the overlying plasma membrane, increasing intracellular Ca2+ levels. This increase in intracellular calcium levels activates phospholipase C (PLC), which hydrolyses phosphatidylinositol biphosphate (PIP2), leading to the release of PIP2-bound tyrosine-phosphorylated-gelsolin, which undergoes dephosphorylation/activation by $Ca2^{+}$ (O'toole et al., 2000). Gelsolin is an actin-severing protein which causes fast F-actin depolymerisation before the acrosome reaction. The zona pellucida and progesterone from the cumulus cells cause an influx of $Ca2^{+}$ increasing intracellular Ca2 + concentrations in the sperm tail, directly and indirectly, stimulating acrosome reaction, respectively (O'toole *et al.*, 2000).

2.3.2 FERTILISATION

Fertilisation is a complex multistep molecular process involving three unique events: (i) the movement of spermatozoa through the cumulus matrix, (ii) the attachment and penetration of spermatozoa through the zona pellucida, and (iii) the union of the two plasma membranes (Kaingu et al., 2016).

The ovulated ovum is usually surrounded by a layer of loosely packed follicular cells known as the cumulus oophorus. As the sperm approaches the cumulus oophorus, it releases the enzyme *hyaluronidase,* which dissolves hyaluronic acid that binds the cells of the cumulus oophorus together. This dissolution of hyaluronic acid causes the cumulus oophorus cells to disperse, leaving space for spermatozoa to swim through to reach the zona pellucida (E. Kim et al., 2008).

The zona pellucida is an extracellular matrix composed of three glycoproteins: ZP1, ZP2, and ZP3 (Jones & Lopez, 2014). Receptors on the sperm plasma membrane attach to ZP3, allowing the sperm to adhere to the zona pellucida (Jones & Lopez, 2014). This binding triggers the sperm head to undergo the acrosome reaction (Jones & Lopez, 2014). An influx of calcium and a rise in pH and cAMP levels within the sperm head cause exocytosis of the acrosomal vesicle (Jones and Lopez, 2014). Exocytosis occurs when the plasma membrane of the sperm fuses with the outer acrosomal membrane, forming many small openings to the acrosome (Jones & Lopez, 2014). The acrosome contains hydrolytic enzymes that spill out and degrade the zona pellucida near the sperm head. This forms a tunnel in the zona, through which the sperm begins to move (Jones & Lopez, 2014).

First, there is the attachment of the spermatozoa to the zona pellucida, followed by the acrosome reaction, which enables the spermatozoa to penetrate the zona pellucida ending up with the fusion of the sperm and egg pronuclei (Kaingu et al., 2016).

Once the spermatozoa penetrate the zona pellucida, it approaches the egg sideways instead of head-on (Jones & Lopez, 2014). The sperm head lies parallel to the egg cell surface within the narrow perivitelline space (Jones & Lopez, 2014). At this point, the posterior part of the sperm head attaches to the egg plasma membrane (Jones & Lopez, 2014). The sperm and ovum plasma membranes then fuse, forming an opening into which the sperm nucleus, midpiece, and

most of the tail sink into the egg cytoplasm. After penetrating the egg, the sperm chromatin undergoes dramatic changes of decondensation followed by recondensation, where the DNA is restructured. After sperm chromatin decondensation and recondensation, sperm and egg haploid genomes form pronuclei and move toward each other; their membranes interdigitate and disperse to facilitate nuclear union (Wu and Dean, 2020).

After the sperm fertilises the ova, available free calcium is suddenly released in the egg cytoplasm from its cytoplasmic storage (Cortical reaction). This rise in calcium triggers the cortical granule membranes to fuse with the adjacent cell membrane (Jones & Lopez, 2014). Thus, the cortical granules open to the exterior and release their contents into the perivitelline space (Jones & Lopez, 2014). The cortical granules include enzymes that act on components of the zona pellucida. These enzymes alter ZP2 and ZP3, destroying their receptor sites for the sperm head (Jones & Lopez, 2014). Consequently, this prevents additional sperm from attaching to the zona pellucida and gaining access to the ovum (Jones & Lopez, 2014). Complex spermatozoa-ova interactions are required for successful fertilisation (Sánchez and Smitz, 2012).

2.3.3 PREIMPLANTATION EMBRYO DEVELOPMENT

Once the spermatozoon penetrates the ovum, the oocyte arrested in metaphase II is activated to finish meiosis II. This trigger results from the changing levels of calcium during the zona reaction (Namiki et al., 2018). The embryo traverses the oviduct and enters the uterus at which stage is now referred to as a blastocyst.

Following fertilisation, the zygote undergoes several cleavage divisions that divide the ooplasm into smaller compartments known as blastomeres. The mitotic divisions continue until the embryo undergoes compaction, or intracellular adhesion, as the first to form a morula.

It is believed that the “closing and opening” of the sphincter at the isthmus-uterine junction sympathetic neuronal circuitry is under the control of ovarian hormones, thereby regulating the timely transit of embryos from the oviduct into the uterus (Wang et al., 2006).

In the mouse, compaction occurs after the third cleavage division. Compaction is the first morphogenetic event. This event varies considerably among mammalian species, e.g., in bovines, during the 32-64 embryo stage (Betteridge and Fléchon, 1988). During compaction, interblastomeric contact increases, which ultimately veils the individual blastomeric outlines of the early embryo until the embryo appears as a uniform cellular mass, known as a morula.

Blastocyst expansion causes the zona pellucida to thin out, and this eventually causes the pellucida to rupture, allowing the blastocyst to hatch from the zona pellucida. Blastocyst expansion also allows a large volume of fluid entering the space to shift a grouping of cells off to one side of the curved-out interior to form the inner cell mass.

The zona pellucida is necessary for the development of viable oocytes, fertilisation (e.g., gamete recognition at the zona pellucida), prevention of polyspermy and the protection of early embryos before implantation (Conner et al., 2005; Pang et al., 2011; Wassarman, 2008; Kaingu et al., 2016; Wassarman and Litscher, 2022). After shedding the zona pellucida, the blastocyst becomes fit for implantation (Sánchez and Smitz, 2012). As a result, shedding the zone pellucida or interfering with its structural integrity will interfere with the transfer of nutrients and other molecules through the gap junctions that transverse the zona pellucida (Kaingu et al., 2016). Several anti-zona pellucida vaccines have been developed to help control animal populations.

Pro-inflammatory and anti-inflammatory cytokines are intimately involved in blastocyst hatching and implantation. Hatching-associated proteases are stimulated by growth factors such as EGF, HB-EGF and LIF (Seshagiri et al., 2016). It is observed in several species that

the expression and function of cyclooxygenase-2 (COX-2) and PGs are essential for peri-hatching blastocyst development, including hatching. Mice deficient in cytokines exhibit different reproductive phenotypes, predominantly implantation defects (Dimitriadis et al., 2005; Guzeloglu-Kayisli et al., 2009). For example, knockout phenotypes for cytokines such as IL-1, IL-6, LIF and IFN-c show significant implantation failure or diminished implantations, while others such as GM-CSF, IL-5, IL-10, CSF-1 show only a moderate or no effect on implantation (Evans Wood et al., 2020; Robertson, 2007; Sakurai et al., 2012).

2.3.4 DECIDUALISATION

Decidualisation refers to the differentiation of the elongated stromal fibroblasts into secretory, epithelioid-like decidual cells (Okada et al., 2018). In rodents, this process is inducted by the implanting blastocyst. In humans, decidualisation is independent of the blastocyst signal; it starts immediately after ovulation, reaching the peak in the mid-luteal phase of the menstrual cycle (Gellersen & Brosens, 2014; S. Kim & Kim, 2017). The mice blastocyst reaches the endometrium on the fourth day after fertilisation. Once it attaches to the endometrial lining, the process of decidualisation is triggered. The transformation of fibroblastic stromal cells into decidual cells helps them acquire secretory and biosynthetic properties. The following three days after the onset of decidualisation, the cells proliferate and differentiate substantially, becoming larger in size and having bi-nucleate or polypoid status (Ramathal et al., 2010).

During decidualisation, the vasculature surrounding the zone of the implanting embryo undergoes significant remodelling. This results in an extensive angiogenic network whose function is to support the growth and development of the implanted embryo. These endometrial changes happen in response to progesterone.

During decidualisation, there is an increased production of Insulin-like growth factor-binding protein-1 (IGFBP-1), Prolactin (dPRL), and Forkhead transcriptional factor (FOXO1) in

response to hormonal stimulation (Dey et al., 2006). The expression of decidual-specific genes that encode these proteins requires cAMP. Endometrial cells proliferate vigorously during the follicular phase and decidualisation. The epithelium then differentiates to become secretory. They attain maximal secretory activity 5 to 7 days after the LH surge. In non-pregnant cycles, these glands acquire a spent, serrated appearance (King, 2000). In decidua, copious glandular secretions continue in the decidua spongiosa (the basal third of the mucosa), where there is little intervening stroma, and the appearance of the epithelium looks like that of a fern (King, 2000). In the upper two-thirds of the mucosa (decidua compacta), the glandular epithelium becomes attenuated and non-secretory making it challenging to differentiate glands from vessels without immunostaining for endothelial cells and keratin.

Decidualisation lasts up to day 10.5 of gestation in mice, coinciding with the end of the invasive period of implantation. During differentiation, stromal cell polyploidy eventually leads to apoptosis which limits the lifespan of the decidual cells (Ramathal et al., 2010).

2.4 MATERNAL RECOGNITION OF PREGNANCY

Fertilisation success is generally high after mating. However, a substantial proportion of the resulting embryos do not develop to term, most of which are lost between fertilisation and maternal recognition of pregnancy (Lonergan et al., 2016). Maternal recognition of pregnancy (MRP) refers to the intrinsic signals between the maternal endometrium and the embryo required to maintain the functional lifespan of the corpora lutea (CL), which produces progesterone (Bazer et al., 2017; Bazer and Spencer, 2011). “Progesterone is essential for a quiescent uterine environment that supports implantation, placentation, and development of viable offspring” (Bazer *et al.,* 2017). Different species-specific hormone cytokine signals are needed to recognise pregnancy (Bazer *et al.,* 2017) successfully.

In ruminants, the maternal recognition of pregnancy agent is interferon tau (IFNτ) (Thatcher et al., 2001). IFNτ is unique to the ruminants and is produced by the developing conceptus. It acts on the endometrium in a paracrine manner to inhibit luteolysis, thus sustaining the corpus luteum and progesterone production.

In the sow, the MRP agent is oestrogen. A minimum of two embryos are needed to successfully establish a pregnancy, with at least one embryo in each uterine horn to avert the production of PGF2α. In the absence of an embryo, the uterine horn produces PGF2, which goes into circulation resulting in lysis of the corpus luteum.

In the mare, maternal recognition of pregnancy signals remains unknown. However, there is a migration of the conceptus between the two uterine horns from when it enters the uterus (day six post-ovulation) until fixation on day 18. Peristalsis within the endometrium and the bi-partite nature of the mare uterus appear like physiological facilitators of this movement. This migration stimulates anti-luteolytic activity, reducing PGF2 levels in uterine fluids and venous circulation (Bazer et al., 2017).

There is a scarcity of information on MRP or early pregnancy factors in dogs and cats compared to other species (Raheem, 2017). This may be because MRP is not essential for the sustenance of pregnancy in these species. Whether the bitch/queen is pregnant or not, the CL is maintained for about 60 days (Raheem, 2017).

In humans, the MRP signal is through chorionic gonadotropin (hCG) (Bruner, 1951; Clements et al., 1976). hCG is luteotropic and is produced by the blastocyst on days 4–5 once the embryo descends into the endometrium from the oviduct.

In rodents (mice, rats, and hamsters), maternal recognition of pregnancy involves signalling for the formation of functional CL that can produce P4 to support uterine decidualisation, implantation, and pregnancy (Bazer *et al.*, 2017). The corpus luteum secretes progesterone for

two days and needs vaginal stimulation to become fully functional (Bazer *et al.,* 2017). The lack of a proper luteal phase in non-mated rodents allows them to cycle again to achieve mating and establish a pregnancy. PGF2α is luteolytic in rodents, but its mode of action is not known (Bazer *et al.,* 2017). Cervical stimulation or sterile mating of rodents during oestrus can result in functional corpus luteum. However, this corpus luteum will only secrete progesterone for 12 -14 days; a period referred to as pseudopregnancy. Viable conceptuses within the uterus are required to extend the lifespan of the corpus luteum beyond the twelfth day. Two endocrine events are needed to maintain pregnancy in rodents: Mating/cervical stimuli provoke semi-circadian prolactin surges from the pituitary (Bazer et al., 2017; Osada et al., 2001). Prolactin increases LH receptors on luteal cells to form a functional corpus luteum. It also suppresses AKR1C1 (20α-HSD) activity within the corpus luteum, preventing progesterone conversion to inactive 20α-hydroxyprogesterone (Bazer et al., 2017). Secondly, implantation, conceptus growth, and production of lactogenic hormones by placentae and uterine decidua also help maintain the corpus luteum beyond day 12 (Spencer and Bazer, 2004). These members of the lactogenic family of hormones take over from pituitary PRL as luteotropic hormones that act on CL to support the production of P4 throughout gestation.

2.5 IMPLANTATION

Implantation is the process where the blastocyst makes intimate physiological and physical contact with the endometrium. The quality of the blastocyst, the presence of a receptive endometrium, and the synchronisation of the embryo's developmental stages all contribute to successful implantation (Kaingu et al., 2016). Successful implantation is dependent on the availability of a competent embryo and a receptive uterus (Wang *et al.,* 2006). ‘The early embryo enters the uterine cavity as a morula and becomes a 32 to 256-cell blastocyst before implantation (Kim and Kim, 2017). Implantation begins with the loss of the zona pellucida (hatching) about 1-3 days after the morula enters the uterine cavity in preparation for

attachment (Kim & Kim, 2017). The active blastocyst undergoes structure changes such that a more irregular surface with more microvilli is observed with the accumulation of glycogen granules in the cytoplasm' (Kim and Kim, 2017).

Implantation is a complicated process involving the coordinated actions of autocrine, paracrine, and endocrine systems and is influenced by various factors, some of which are still unknown (M. Singh et al., 2011). As a result, implantation failure might be caused by poor blastocyst quality or endometrial receptivity (Kaingu et al., 2016). Animal models have revealed important information about the molecular pathways during embryo implantation (Dey et al., 2004; Wang et al., 2003). The growth of an embryo to the blastocyst stage (Kubota et al., 2016) and the formation of an endometrium that is receptive to the embryo are both required for successful implantation (Kaingu et al., 2016; Kubota et al., 2016). In rodents and humans, the endometrium is known to become responsive for only a brief duration known as the 'implantation window.' The embryo cannot correctly establish contact with a refractive endometrium once this period of receptivity has passed.

After fertilisation, hormones and growth factors acting locally mediate the initial communication between the free-floating blastocyst and the receptive endometrium (Kaingu et al., 2016; Lopata et al., 2002). When the endometrium is exposed to estrogen and progesterone in a controlled manner during the ovulatory cycle, the endometrium becomes 'decidualised' in the second half of the cycle, allowing the embryo to implant. Decidualisation, which requires both estrogen and progesterone, is irreversible and results in programmed cell death (apoptosis) if implantation does not occur. As a result, progesterone and estrogen mobilise several molecular modulators that aid embryo implantation (Lim et al., 2002).

2.5.1 CLASSIFICATION OF IMPLANTATION

Implantation may be invasive or non-invasive depending on how deep the embryo trophectoderm embeds into the uterine luminal epithelium stroma (Bazer et al., 2010). Bonnet (1884) classified implantation into three categories based on distinct blastocyst-uterine cell-cell interactions: central, eccentric, and interstitial (Cha et al., 2015; Dey et al., 2004; Dey & Lim, 2006). Central implantation occurs in mammals such as ferrets, rabbits, and some marsupials. In these animals, blastocysts develop and expand considerably before implantation (Cha et al., 2015; Dey et al., 2004; Dey & Lim, 2006). In contrast, the blastocysts in mice, rats, and hamsters are small and exhibit modest expansion (Dey et al., 2004). In these species, an implantation chamber is formed via the invagination of the uterine epithelium, an attribute of eccentric implantation (Dey et al., 2004). In guinea pigs, chimpanzees, and humans, implantation is of the interstitial type, i.e., blastocysts are embedded inside the subepithelial stroma (Cha et al., 2015; Dey et al., 2004).

Enders and Schlafke (1967) classified implantation based on ultrastructural studies into intrusive, displacement, and fusion types (Cha et al., 2015; Dey et al., 2004; Dey & Lim, 2006). In invasive implantation, which happens in guinea pigs and humans, the trophoblasts penetrate via the luminal epithelium, achieving and extending through the basal lamina (Dey et al., 2004; Dey & Lim, 2006). The displacement kind of implantation happens in rodents; the luminal epithelium is freed from the underlying basal lamina, facilitating the unfolding of trophoblasts via the epithelium (Dey et al., 2004). The fusion kind of implantation, in which trophoblasts connect with the luminal epithelium by forming a symplasma, occurs in the rabbit (Cha et al., 2015; Dey et al., 2004). In many rodents, including mice and rats, implantation constantly occurs at the uterus's antimesometrial aspect, whereas in some bats, implantation is mesometrial (Cha et al., 2015; Dey et al., 2004; Dey & Lim, 2006). The embryos elongate in different animals and connect over the whole endometrium (pig, horse, and wallaby) or solely

at specialised areas known as caruncles (cow and sheep) (Dey et al., 2004). The attachment response coincides with a localised increase in stromal vascular permeability at the blastocyst, as can be tested by using an intravenous injection of a macromolecular blue dye (uterine blue reaction) (Dey et al., 2004; Dey & Lim, 2006). The first sign of the attachment response (apposition stage) in the implantation process occurs in the mouse and rat on the evening of day four and day 5, respectively, and on day 6.5 in the rabbit (Cha et al., 2015; Dey et al., 2004; Dey & Lim, 2006). In primates, the attachment response happens approximately on day 8 in humans and baboons, on day 9 in macaques, and on day eleven in marmoset monkeys (Cha et al., 2015; Dey et al., 2004; Dey & Lim, 2006). In large domestic animals, the first symptoms of attachment show up on day13 in pigs, day 20 in cows, day sixteen in sheep, and day 19 in goats (Cha et al., 2015; Dey et al., 2004; Dey & Lim, 2006). In mice and humans, stromal cells surrounding the implanting blastocyst endure decidualisation, eventually embedding the embryo into the antimesometrial stromal bed (Cha et al., 2015) .

In mice, blastocysts are oriented with ICMs directed mesometrially, whereas, in people, ICM is directed antimesometrially (Cha et al., 2015; Dey et al., 2004; Dey & Lim, 2006). The mechanisms that control the blastocyst to the antimesometrial luminal epithelium or through which the blastocyst is oriented (Dey et al., 2004; S. Kim & Kim, 2017). There is proof that in progesterone-treated delayed implanting mice, blastocysts are positioned antimesometrially, and interdigitation (apposition) of luminal epithelial telephone microvilli takes place with these the abembryonic or lateral trophectoderm cells of the blastocyst with its ICM oriented towards the uterine lumen (Cha et al., 2015; Dey et al., 2004; Dey & Lim, 2006; Yoshinaga & Adams, 1966). This observation led to the advice that blastocysts maintain the orientation they adopted throughout delay upon initiation of the attachment response and, consequently, the implantation process via estrogen (Dey et al., 2004). During everyday implantation in mice with the onset of luminal closure, blastocysts are positioned at the antimesometrial aspect of

the lumen alongside the uterine axis (Dey et al., 2004; Cha et al., 2015; Yoshinaga, 2018). Shortly after the luminal closure, zona-encased blastocysts are placed in implantation chambers with random orientation of the ICMs (Dey et al., 2004; Plant et al., 2014). However, blastocysts are efficaciously oriented with their ICMs directed at the mesometrial pole by establishing the attachment reaction (Dey & Lim, 2006). This observation suggested that the trophectoderm of the entire blastocyst surface is achievable for attachment to the luminal epithelium (Cha et al., 2015; Dey et al., 2004; Dey & Lim, 2006; H. Singh & Aplin, 2009; Yoshinaga, 2018). That attachment happens randomly immediately after the loss of the zona pellucida (Dey et al., 2004). Evidence presented by Yi et al. (2005) proposes that the blastocyst's proper orientation is achieved through the free motion of the ICM (Dey et al., 2004; Dey & Lim, 2006). However, further investigation is quintessential to unravelling this issue (Dey et al., 2004). From the luminal closure to the attachment reaction, these events occur between and ninety-two hours after coitus in mice (Dey et al., 2004; Cha et al., 2015; Ye, 2020).

2.5.2 STAGES OF IMPLANTATION

Implantation has three stages: apposition, adhesion, and penetration (Dey et al., 2004; Dey & Lim, 2006; Cha et al., 2015). The embryonic trophectoderm cells become closely positioned to the uterine lumen epithelium during apposition (Dey et al., 2004; Cha et al., 2015) . Adhesion involves the trophectoderm cells and the luminal epithelium becoming intimately associated such that the blastocyst cannot be flushed out of the uterine lumen. Penetration consists of the invasion of the luminal epithelium by the trophectoderm (Dey et al., 2004; Dey & Lim, 2006). In mice, embryos enter the uterus at the late morula or early blastocyst stage (Herington et al., 2018; Reese et al., 2008). Here, they hatch from the zona pellucida and implant into the receptive uterus (Wang et al., 2006). Regular transport through the oviduct is required for timely embryo implantation in the uterus (Wang et al., 2006). Any interference in this process

will result in embryo retention in the oviduct, increasing the pregnancy failure rate (Wang et al., 2006).

In mammals, specifically rodents, generalised stromal oedema happens before the starting of apposition (Lee et al., 2007). This event leads to the closure of the uterine lumen, which results in interdigitation of the microvilli of the trophectoderm and the luminal epithelia (apposition), accompanied by nearer contact between them (the adhesion or attachment reaction) (Dey et al., 2004; Cha et al., 2015). Luminal closure occurs at some stage in the whole uterus for the duration of pregnancy or pseudopregnancy, indicating that it does not require the presence of blastocysts (Zhang et al., 2013). Priming the uterus with progesterone seems adequate for this match; luminal closure is impeded in the absence of progesterone (Dey et al., 2004; Young, 2013). Luminal closure and apposition occur in progesterone-treated delayed implanting mice, but the attachment reaction does not occur . Attachment requires estrogen treatment to appear (Dey et al., 2004; Dey and Lim. 2006; Young, 2013; Cha et al., 2015.

In conclusion, the window for successful implantation could be defined as a limited period when the activated stage of the blastocyst is superimposed on the receptive state of the uterus (Kim and Kim, 2017; Paria et al., 1993).

Acetaminophen's conversion to AM404 inhibits the reuptake of anandamide, increasing the levels of anandamide in the extracellular space, which may negatively affect the implantation process. In this study, due to limited resources, it was impossible to measure the levels of anandamide. Still, we correlated the events of the implantation process between treatment and control groups and attributed them to these levels.

2.5.3 PLACENTATION

'The normal mammalian placenta is an apposition or fusion of the fetal membranes to the uterine mucosa for physiological exchange'(Dey et al., 2004; Mossman, 1991). The placenta

is the first and largest fetal organ to develop in mammals. The placenta and associated extraembryonic membranes have identical genetic make-up as the fetus since they are derived from the zygote during early pregnancy (Burton and Fowden, 2015). The two key tissue sources are the trophectoderm and the underlying extraembryonic mesoderm.

Following implantation, the trophectoderm (TE) cells continue to proliferate and form extra-embryonic ectoderm and ectoplacental cone. Trophectoderm will give rise to the placenta, while the ICM gives rise to the embryo and visceral endoderm (Maltepe and Fisher, 2015; Sasaki, 2010). In mice, each blastomere is totipotent and capable of giving rise to either ICM or TE (Maemura et al., 2021). However, it is considered irreversible once the commitment to TE or ICM occurs (Maltepe and Fisher, 2015). Gastrulation in mice occurs around day 6.5 of gestation generating the extra-embryonic mesoderm cell line (Tam and Behringer, 1997). This essential layer originates from the epiblast and forms the allantois and the extra-embryonic mesodermal layer of the amnion and chorion (Hemberger et al., 2020).

Gastrulation starts at gestation day 6.5, forming the amnion, chorion and allantois (Hemberger et al., 2020). ‘At the posterior pole of the embryo, the emerging primitive streak (PS) leads to the ingression of the mesoderm between the endoderm and ectoderm to establish the three germ layers. Chorio- allantoic fusion allows mesoderm-derived blood vessels to invaginate into the chorionic trophoblast layer to form the placental labyrinth. Between gestation days 10 and 14.5, the labyrinthine architecture becomes increasingly intricate to facilitate nutrient and gas supply to the embryo. Syncytial- trophoblast (STB) cells arising from the fusion of chorionic trophoblast cells establish the main exchange surface of the placental labyrinth for nutrient transport. Trophoblast giant cells (TGCs) line the implantation site and are in direct contact with the maternal decidua. Together with the spongiotrophoblast (SpT) and the developing glycogen trophoblast (GlyT) cells, they form the major endocrine compartment of the placenta (Hemberger et al., 2020).’

2.5.3.1 Classification of placenta

‘Mammalian placentas are divided into two according to the fetal membrane including the chorion, yolk sac placenta (choriovitelline placenta) and chorioallantoic placenta’ (Furukawa et al., 2014). The vascularised trilaminar yolk sac attached to uterine tissue forms the yolk sac placenta, which often functions as a transitory placenta in the early post-implantation stage before the allantoic circulation is established (Furukawa et al., 2014). Except in rats and rabbits, most animals lose their yolk sac placentas after the first trimester. The main placenta in mammals throughout mid to late pregnancy is the chorioallantoic placenta, which develops from the dam's endometrium and the embryo's trophectoderm (Furukawa et al., 2014). Based on the morphology of the various animal species, it displays a wide range of shapes (Furukawa et al., 2014).

Based on the gross morphology, placentas can be classified as; (i) Diffuse: this type of placenta occurs over the entire surface of the uterine luminal epithelium with the formation of folds/villi and is found in horses and pigs (Furukawa et al., 2014). (ii) Cotyledonary: this type of placenta is characterised by many spot-like placental regions of the endometrium known as caruncles (from 100 to 120 caruncles in sheep and four caruncles in deer). Intervening areas of the chorion are smooth and relatively avascular (Furukawa et al., 2014). This type of placenta is found in ruminants. (iii) Zonary. This type of placenta shows an intimate interdigitating contact zone that forms a belt around the chorionic sac (Furukawa et al., 2014). This type of placenta is found in carnivores. (iv) Discoid: this type of placenta is characterised by a single (discoid) or double disc (bidiscoid), and interaction is confined to a roughly circular area (Furukawa et al., 2014). This type of placenta is found in primates, rodents and rabbits (Furukawa et al., 2014).

Epitheliochorial type: this type is the most superficial placenta and lacks significant invasion of the uterine lining (Dey et al., 2004; Furukawa et al., 2014). Pockets of columnar trophoblasts

are loosely applied to the maternal endometrial epithelium (Dey et al., 2004; Furukawa et al., 2014). No destruction or invasion of the maternal tissues occurs, and no layers are removed (Furukawa et al., 2014). The epitheliochorial type is found in horses, pigs and ruminants (Furukawa et al., 2014). Endotheliochorial type: the maternal uterine epithelium and connective tissue disappear after implantation, and the trophoblasts come into direct contact with the maternal endometrial (Furukawa et al., 2014). The endotheliochorial type occurs in orders from all four classes of eutherian mammals, including carnivores(Enders and Carter, 2012; Furukawa et al., 2014). Hemochorial type: this type is the most invasive placenta. All maternal tissue layers disappear through erosion, leading to a direct connection between the chorion and maternal blood (Dey et al., 2004; Furukawa et al., 2014). There are haemomonochorial (primates), hemodichorial (rabbits), and hemotrichorial (rats and mice) placentas, with one, two and three trophoblast layers, respectively (Takata et al., 1997; Furukawa et al., 2014).

2.5.3.2 Functions of the placenta

In addition to nutrient and gas exchange, the placenta acts as an endocrine organ producing progesterone, estrogen and prostaglandins at various stages of gestation. The corpus luteum initially produces progesterone in all mammals. In some species, the corpus luteum remains the only relevant source of progesterone throughout pregnancy with minimal (goat, pig) or no (dog) placental contribution (Schuler et al., 2018). In contrast, the placenta adopts this role in other species after a species-specific gestation time. The luteo-placental shift occurs in sheep, horses, man (Meyer, 1994; B. F. Mitchell and Taggart, 2009).

2.6 ENDOCRINE CONTROL OF PREGNANCY

Pregnancy is the period of reproduction during which a female carries one or more live offspring from implantation through gestation in the uterus. It begins when a fertilised zygote

implants in the female's uterus and ends once it leaves the uterus (parturition). Pregnancy is complex and irreversible. It comprises discrete events, including implantation, decidualisation, placentation and finally, parturition (Cha et al., 2012). The success of each event is essential to advance toward the next stage. There is a high occurrence of fetal deaths in mammals and humans; about 66% failure rate of pregnancies in healthy women trying to conceive (Roberts et al., 1996). Most fatalities occur before or during implantation, with a higher percentage of embryonic wastage following assisted reproductive technologies (Roberts et al., 1996).

Several external cues, including cytokines, growth factors, nutrients and metabolites, affect the rate of development and specific cellular events in the early embryo across species. Uterine glands are essential for the establishment of pregnancy and have critical roles in endometrial receptivity to blastocyst implantation, stromal cell decidualisation, and placentation.

2.6.1 PROGESTERONE

The biological effects of progestins and oestrogens, as well as steroidogenesis in the maternal, placental, and fetal compartments, are crucial before and during pregnancy. The crucial roles of progesterone in implantation and parturition have been demonstrated. These roles include promoting endometrial decidualization, inhibiting smooth muscle contraction, decreasing prostaglandin (PG) synthesis, which helps preserve myometrial quiescence and delay the commencement of uterine contractions, and inhibiting immunological responses resembling those involved in graft rejection.

Progesterone is an endogenous steroid produced by the adrenal cortex, ovaries and testis. Progesterone is the hormone of pregnancy. The corpus luteum produces it during the first few weeks of pregnancy before production is shifted to the placenta. However, in some species like the ovine, progesterone production is mainly from the corpus luteum. Progesterone helps maintain a quiescent uterus establishing a conducive environment for embryo implantation,

growth and development. It also helps decrease the level of vascular tone in the myometrium and prevent myometrial contractions that may impair implantation. In addition, it causes an influx of inflammatory cells within the endometrium, enhancing immunity during pregnancy (immunotolerance). Progesterone also acts as a vasodilator, reducing blood pressure during pregnancy. Women who suffer from pre-eclampsia have been shown to have low progesterone levels and have been successfully treated using synthetic progesterone (Amaral et al., 2014).

There are two primary progesterone receptor (PR) isoforms in reproductive tissues, nuclear PR (NPR) isoforms PRA and PRB. PRA primarily functions as a ligand-dependent trans-dominant repressor of PRB activity. PRB is thought to mediate myometrial quiescence during pregnancy. The ratio of PRA to PRB is essential in determining the status of P4 signalling. An increase in PRA: PRB ratio is associated with the onset of labour. This increase in PRA: PRB ratio results in increased expression of pro-labour genes such as cyclooxygenase-2 (COX-2), connexin-43 (Cx43), nuclear factor κB subunit 2 (NF-κB2) and oxytocin receptor (OTR) which can stimulate myometrial contractility (Winterhager et al., 2019).

Mice carrying a null mutation of the progesterone receptor gene exhibit several reproductive abnormalities, including anovulation, attenuated lordotic behaviour, uterine hyperplasia, and lack of mammary gland development (Chappell et al., 1997; Dey et al., 2004; di Renzo et al., 2012). PR null rats are infertile due to deficits in sexual behaviour, ovulation, and uterine endometrial differentiation (Kubota et al., 2016). In contrast to PR null mice, PR null rats did not have disruptions in the oestrous cycles.

Studies using PR-null mouse strains have demonstrated that uterine stromal cells are the mediators of the inhibitory effects of progesterone on the oestrogen-induced proliferative response of the uterine epithelium (Robertshaw et al., 2016b).

Progesterone stimulates the release of leukaemia inhibitory factor (LIF) through interleukin (IL)-4, which has also been demonstrated to promote implantation and pregnancy continuation (Dey et al., 2004; Maccarrone et al., 2001). Progesterone insufficiency has been shown to cause miscarriage across the species. Low levels of progesterone cause infertility due to the loss of these essential endometrial changes, resulting in an impaired ability of the endometrium to allow for proper embryo implantation.

2.6.2 OESTROGEN

Oestrogen plays a vital role in the changes observed within the uterus during early pregnancy (Robertshaw et al., 2016). In mice, there are two phases of oestrogen secretion; in phase 1, oestrogen secretion occurs during the pre-ovulatory period, and in Phase 2, oestrogen secretion occurs during the pre-implantation period. Phase 1 oestrogen secretion takes place during the first two days of gestation; this oestrogen secretion stimulates the proliferation of the luminal and glandular epithelial cells (Robertshaw et al., 2016). The pre-implantation oestrogen secretion potentiates the effects of progesterone on the endometrium, stimulating stromal proliferation. Studies in mice ovariectomised on the morning of the day4 before the preimplantation, oestrogen secretion resulted in delayed implantation due to blastocyst dormancy (Robertshaw et al., 2016; Yoshinaga & Adams, 1966a). When the uterus was exposed to progesterone alone, they were rendered neutral or pre-receptive for implantation and receptivity was only restored after exposure to oestrogen (Paria et al., 1993). This demonstrates the critical role of oestrogen in implantation (Robertshaw et al., 2016).

Phase 2 oestrogen secretion takes place during implantation on day 4 of pregnancy. After forming the corpus luteum on the third day of pregnancy, progesterone produced stimulated stromal cell proliferation. This action of progesterone is potentiated by phase 2 oestrogen on the day of implantation, i.e., day 4. This second oestrogen surge before implantation stops epithelial cell proliferation and allows differentiation (Robertshaw et al., 2016). At this time,

the uterine epithelium undergoes remodelling, and there is a downregulation of the cell-to-cell adhesion molecule E-cadherin, making the cells lose polarity (Robertshaw et al., 2016). In addition, the epithelial cells acquire inhibition of glycoprotein mucin 1 (Surveyor et al., 1995). This allows the cells to develop protrusions along their apical surface. At the location of the blastocyst is also exhibited there is increased endometrial capillary permeability leading to implantation and subsequent decidualisation of stromal cells.

Oestrogen exerts its action on target organs through oestrogen receptors (ER). Two oestrogen receptors, ER and ER, act as ligand-inducible transcription factors(Tsai and O'Malley, 2003). The expression and distribution of these receptor subtypes vary depending on their tissue-specific physiological functions. ERα is found in the mammary gland, uterus, theca cells, bone liver, adipose tissue, testes, epididymis and prostate stroma, while ERβ is located in the prostate epithelium, granulosa cells, bladder, colon, adipose tissue and the immune system (Dahlman-Wright et al., 2006; Heldring et al., 2007). Both receptors have a high affinity to oestradiol-17 β (E2) in the same oestrogen response element (ERE). They share approximately 95% homology in the DNA-binding domain and 55% in the hormone-binding domain. There is the compartmentalisation of uterine Erα during early gestation. On gestation days 1 and 2, Erα mRNA is predominantly localised in the luminal and glandular epithelium. Additionally, localisation is seen in the stroma on days 3 and 4; however, by day 8 of gestation, Erα exhibits downregulation of decidual cells immediately surrounding the embryo (Tan et al., 1999). During early peri-implantation days of pregnancy, there is a highly low-to-undetectable expression of Erβ (Robertshaw et al., 2016).

Biological disruptions in the ER genes have been associated with infertility. Specifically, disruption of the Erα gene causes infertility due to defects in the reproductive tract and gonads of female mice (Eddy et al., 1996; Lubahn et al., 1993), whereas disruption of the Erβ gene is linked to impaired ovulation (Couse et al., 2005; Krege et al., 1998; Robertshaw et al., 2016).

Erα is essential for endometrial receptivity (Lubahn et al., 1993). These studies imply that precise regulation of ER gene expression seems to define the implantation window.

Evaluation of the implantation window has shown that the effects of oestrogen on the endometrium are highly regulated. Ma et al., (2003) demonstrated that lower oestrogen levels are required to sustain the receptivity of the uterus, whereas higher concentrations shut down the implantation window. However, the exact mechanism is not well understood. Dysregulation of oestrogen production or expression of oestrogen receptors can lead to implantation failure through the inability to obtain a receptive uterine epithelium.

2.6.3 GROWTH FACTORS AND CYTOKINES

Mammalian preimplantation embryos develop along the reproductive tract without direct cellular contact with the oviduct or uterus until implantation. Growth factors derived from early embryos and maternal reproductive tracts play essential roles during preimplantation embryo development. These include insulin-like growth factors (ILG-F), epidermal growth factors (EGF), fibroblast growth, and platelet-derived growth factors.

A wide range of cytokines also influences the development of the preimplantation embryo (Robertson et al., 2015). These cytokines can be classified according to their effect on the embryo (embryotrophic or embryotoxic) and by their source as autocrine, paracrine or endocrine. Female tract tissues also secrete almost all of the cytokines synthesised endogenously within the embryo, so there is an opportunity for both autocrine and paracrine activity (Robertson et al., 2015). However, some cytokines, notably embryotrophic factors GM-CSF and CSF1, are not synthesised by the embryo and are entirely maternal in origin (Robertson et al., 2015).

2.6.4 EMBRYOTROPIC FACTORS

2.6.4.1 Insulin-like growth factor

Insulin exerts trophic effects on the early embryo; the early embryo expresses a functional receptor for insulin but not insulin itself. Insulin-like growth factor (IGF) is an endocrine signal that has been demonstrated to have a thermoprotective (Bonilla et al., 2011; Jousan and Hansen, 2004) and anti-apoptotic effect (Spanos et al., 2000) on the developing embryo. IGF-1 stimulates granulosa cells' estrogen production by enhancing FSH and LH's impact on the follicular cells. Indeed, IGF-1 knock-out mice survive to adulthood but are infertile due to impaired steroidogenesis and reduced FSHR mRNA levels within the granulosa cells. Insulin-like growth factor 1 (IGF-1) is an essential endocrine signal regulating early embryonic development. And can enhance the survival of embryos after transfer to recipients.

Insulin-like growth factor increases the percentage of preimplantation embryos that transition to blastocysts and modifies blastocyst gene expression, increasing the resistance of embryos to different stresses (Bonilla et al., 2011).

2.6.4.2 Epidermal growth factor

Epidermal growth factor (EGF) is a common mitogenic factor that stimulates the proliferation of different types of cells, especially fibroblasts and epithelial cells. EGF activates the EGF receptor (EGFR/ErbB), which initiates, in turn, intracellular signalling. Epidermal growth factors promote the nuclear and cytoplasmic maturation of IVG oocytes during meiotic development. Although the exact function of EGFs is unknown, the EGF receptors are expressed on stromal cells during pregnancy, suggesting a role as paracrine mediators driving stromal proliferation (Song et al., 2000; Xie et al., 2007).

2.6.4.3 Platelet-activating factor

The embryo produces platelet-activating factor (PAF) or 1-o-alkyl-2-acetyl-sn-gylcero-3-phosphocholine soon after fertilisation. PAF acts in an autocrine manner on the source, stimulating metabolism, viability, and cell-cycle progression (O'Neill, 2005). The release of embryo-derived paf causes platelet activation within the microvasculature of the oviduct (Steinand O'Neill, 1994). It is postulated that the activated platelets are likely trapped by the reticuloendothelial system and are therefore lost from circulation resulting in early pregnancy-associated thrombocytopenia observed in hamsters (Velasquez et al., 1995), mice (O'Neill, 1985), rabbits (Kojima et al., 1996) and bovine (Kojima et al., 1996). Humans exhibit mixed reactions of either thrombocytopenia or thrombocytosis (O'Neill, 1990; Yeung et al., 1992). PAF also induces the modification of T-lymphocyte rosette formation (Orozco et al., n.d.; Sueoka et al., 1988), changing the function of the maternal immune system early in pregnancy (O'Neill, 2005). The exact role of this modification remains unclear, although there is evidence of maternal immunosuppression in early pregnancy that could be attributed to PAF production by the developing embryo.

2.6.4.4 Leukaemia inhibitory factor (LIF)

Leukaemia inhibitory factor (LIF) is among the most critical cytokines in the reproductive tract. A pleiotropic cytokine from the interleukin- (IL-) 6 family regulates several cellular functions via binding to membrane-bound LIF receptor (LIFR) and gp130.

Ovarian steroids have been shown to play a vital role in regulating LIF, LIFR, and gp130 expressions within the uterus during the implantation window (Salleh and Giribabu, 2014). The glandular epithelium produces leukaemia inhibitory factor (LIF) under estrogen stimulation (Stewart et al., 1992a).

In order to carry out its biological activity, it first activates its receptor (LIFR), then recruits the glycoprotein 130 (GP130) (Taga and Kishimoto, 2003). Lifr and Gp130 expression patterns in the luminal epithelium on day 4 of mouse pregnancy were revealed by Yang et al. in 1995. The nonreceptor tyrosine kinase Janus kinase (JAK), which LIF activates on the luminal epithelium, mediates the phosphorylation and activation of signal transducer and activator of transcription 3 (STAT3) (Heinrich et al., 1998; Robertshaw et al., 2016b; Tomida et al., 1999).

Studies have shown that LIF is involved in several events during implantation, including transforming the endometrium into a receptive state, driving the interaction between the embryo and endometrium, stromal decidualisation, trophoblast invasion, growth and development of the blastocyst, and uterine leukocyte infiltration (Cullinan et al., 1996; Staun-Ram and Shalev, 2005; Stewart et al., 1992b; Sun et al., 2013a; W. Wang et al., 2012).

In Lif-null mice, ER and PR expression are typical. On day 4 of pregnancy, they still don't express EGF-like growth factors including amphiregulin (Areg), heparin-binding epidermal growth factor (HB-EGF), and epiregulin (Ereg) close to the blastocyst (Song et al., 2000; Robertshaw et al., 2016). Additionally, Stat3-null mice exhibit elevated epithelial expression of the estrogen-regulated genes Ltf and Muc1, which heighten estrogen signaling and permit persistent proliferation in the luminal epithelium and a lack of proliferation in the stromal layer, indicating a nonreceptive uterine state (Sun et al., 2013b).

Without LIF expression in the uterus, blastocyst implantation cannot occur (Stewart et al., 1992b). LIF mRNA is expressed in decidual leucocytes. Forkhead box A2 (FOXA2) is expressed only in the glandular epithelium and regulates its development and function. Mice lacking FOXA2 in the uterus are infertile due to defective embryo implantation arising from a lack of leukaemia inhibitory factor (LIF).

LIF plays a role in the regulation of immunity during early pregnancy. It affects the uterine leukocyte subpopulation by recruiting a specific group of leucocytes to the implantation site. Mice deficient in LIF were found to have many uterine macrophages, while the number of natural killer cells and eosinophils was reduced.

LIF mediates a shift from a proliferative state of the luminal epithelium to a differentiated state through the down-regulation of cell-cell junctional molecules acting as a barrier to embryo invasion (Kim and Kim, 2017). These findings demonstrate that the loss of the LIF-STAT3 signalling pathway culminates in the undifferentiated uterine epithelium and is therefore nonreceptive to embryo implantation (Robertshaw et al., 2016).

2.6.4.5 Colony-stimulating factor-1

Colony-stimulating factor-1 (CSF-1) is a glycoprotein that controls macrophage proliferation, differentiation, and survival, as well as the growth and expansion of extra-villous trophoblasts. It plays a crucial role in embryo implantation and placentation (Robertshaw et al., 2016).

The embryo and endometrium express CSF-1 receptor mRNA, and it has been suggested that cross-talk of endometrial epithelial CSF-1 with trophectodermal CSF-1 receptor enhances attachment (Pollard et al., 1991).

Placental tissue express CF-1 and CF-1R. Mice with an inactivating mutation in the colony-stimulating factor-1 (CSF-1) gene are infertile due to lower rates of implantation and foetal viability.

2.6.4.6 Interleukin 1 and 6

Interleukins (ILs) are polypeptide cytokine factors of the immune system. They were initially defined by their action between leukocytes (Gérard et al., 2004). Proinflammatory cytokines are small signalling proteins released by leukocytes that control innate and adaptive immune

responses (Mathew et al., 2016). They also mediate communication between the early conceptus and endometrium, promoting implantation and the establishment of pregnancy.

Interleukin 1 and 6 are proinflammatory cytokines. They belong to a group of locally acting polypeptides growth factors that are potent stimulators of cell proliferation, differentiation and apoptosis. IL-1 is produced by macrophages, B-cells, large granular lymphocytes, endothelium, fibroblasts, and astrocytes. The primary targets are b cells, T cells, macrophages, endothelium and tissue cells. IL-1 causes lymphocyte activation, macrophage stimulation, increased leukocyte/endothelial adhesion, fever due to hypothalamus stimulation, and release of acute phase proteins by the liver. It may also cause apoptosis in many cell types and cachexia.

IL-6 is produced by B and T lymphocytes, macrophages and fibroblasts. The principal target of IL-6 is B lymphocytes and hepatocytes. IL-6's immediate effects include B-cell differentiation and stimulation of acute phase proteins.

Interleukin1(IL-1) and IL 6 are expressed in the mouse uterus during the first three days of pregnancy (Dey & Lim, 2006). IL-6 stimulates vascular endothelial growth factor (VEGF) expression and regulates Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) (Dey & Lim, 2006; Krüssel et al., 2003). A decrease in endometrial IL6 and LIF mRNAs during early pregnancy in humans is linked with infertility.

2.6.5 EMBRYOTOXIC FACTORS

Embryotoxic cytokines can affect embryonic programming through three different aspects; activation of the cell stress response, which alters gene expression, causing an irreversible impact on the developmental path; epigenetic modification, which causes lasting changes to phenotype and behaviour cell death by apoptosis. Like the trophic factors discussed above, these cytokines are responsive to induction and regulation in the female tract (Robertson et al.,

2015). In particular, it can be elevated during local or systemic infection or other nutritional or environmental stressors (Robertson et al., 2015). In the event of embryo exposure to high levels of these factors, the result may be embryo demise and failure to progress to pregnancy (Robertson et al., 2015).

Some of the embryotoxic cytokines include interferon-gamma (IFNγ), Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), and tumour necrosis factor (TNF), which are regulated by environmental events and can also exert potent death-inducing effects on embryo (Robertson et al., 2015).

2.6.5.1 Tumour Necrosis Factor Alpha (TNF)

TNF is a pleiotropic cytokine exhibiting pro-inflammatory functions, eliciting several different cellular responses (Baker & Reddy, 1999; Robertson et al., 2015). Uterine macrophages and natural killer (NK) cells are the primary sources of TNF in mice and women's reproductive tract and oviductal epithelial cells (Gregersen et al., 2000; Robertson et al., 2015; Zolti et al., 1990). TNF stimulates translocation of the nuclear factor kappa B (NFκB) transcription factors to the nucleus, subsequently triggering transcription of various inflammatory mediators such as IL6 and IL1α (Iwamoto & Konicek, 1997; Robertson et al., 2015).

Mouse preimplantation embryos express the TNF receptor's TNFR1 (p60) form (Pampfer et al., 1994; Robertson et al., 2015) and may secrete TNF under some circumstances. The development of pre-implantation embryos is negatively impacted by TNF. For instance, the presence of TNF increases the proportion of apoptotic blastomeres in rat and mouse blastocysts (Byrne et al., 2007; Fabian et al., 2002). (Pampfer et al., 1997). In diabetic rats, there is an increase in the production of soluble TNF, and this appears to be a major factor in the model's poor fertility (Pampfer et al., 1997). Early pregnancy TNF treatment in mice affects implantation and decreases litter size in both mice and rats (Chaouat et al., 2004). SCF, IGFI,

and IGFII are protective against the inhibitory effects of TNF, which makes it interesting that the balance between pro- and anti-apoptotic factors is likely to be critical in determining the result of TNF exposure (Gbowski et al., 2005).

2.6.5.2 Interferon Gamma (IFNγ)

During the first trimester of pregnancy, the uterine lumen is filled with the pro-inflammatory cytokine interferon-gamma (IFN). It is essential for many different cellular activities, such as inducing apoptosis and activating innate and adaptive immune responses (Boehm et al., 1997). Although IFN plays important roles in normal rodent pregnancy, such as the beginning of endometrial vasculature remodelling angiogenesis at implantation sites and maintenance of the decidual component of the placenta, increased production of this cytokine is harmful. GM-CSF and other cytokines that support blastocyst growth and differentiation are inhibited by IFN (Robertson, 2007), which is in line with research demonstrating that IFN prevents implantation and is harmful to the maintenance of pregnancy during the pre-implantation period (Ashkar and Croy, 1999; Murphy et al., 2009; Yockey and Iwasaki, 2018). The adverse effects of IFNγ are partly exerted directly on embryos, as the IFNγ receptor is expressed on mouse oocytes and preimplantation embryos (Truchet et al., 2001), and IFNγ acts as an inhibitory factor in mouse embryo culture (Haimovici and Anderson, 1993; Robertson et al., 2015, 2018).

2.6.5.3 Tumour Necrosis factor-related Apoptosis-Inducing Ligand (TRAIL)

TRAIL and its receptor TRAILR (also known as death receptor, DR) are death-inducing ligand and receptor pairs that belong to the TNF and TNF-receptor superfamilies, respectively (Ashkenazi and Dixit, 1998). From the 1-cell to the blastocyst stage of murine preimplantation embryo development, TRAIL and TRAILR are expressed, and embryos manipulated to express TRAIL or exposed to TRAIL undergo apoptosis (Dey et al., 2004; Riley et al., 2004). TRAIL is constitutively expressed in mouse oviducts and its expression is lowered throughout the

periconceptional stage in response to signals emerging from the embryo or exposure to the seminal fluid (Bromfield et al., 2014). In oviducts and the uterus, bacterial product lipopolysaccharide (LPS) can increase TRAIL expression, suggesting that TRAIL synthesis may occur during an infection (Menchetti et al., 2018).

Following fertilisation in the oviduct, the embryo undergoes several rounds of mitotic cell division, ultimately forming a morula. At the late morula stage, the embryo enters the uterine lumen. It transforms into a blastocyst that contains a cavity (called blastocoel) with two distinct cell populations, the inner cell mass (ICM) and the trophectoderm (the progenitor of trophoblast cells) (Dey et al., 2004; Dey & Lim, 2006). The preimplantation embryo enters the uterine cavity as a morula and becomes a 32 to 256-cell blastocyst before implantation.

2.7 MAMMALIAN REPRODUCTION AND POPULATION CONTROL

Females of reproductive age experience cycles of hormonal activity that repeat at regular intervals. With every cycle, the female body prepares for a potential pregnancy, whether intended or not. For several decades efforts to control domestic and wildlife animals have been going on. A rise in the number of certain wildlife and domestic animal species poses and causes serious conservation problems. These challenges are similar to those experienced due to the escalation in human populations.

Dogs have a global distribution and have developed close and positive associations with people; hence controlling their populations is a challenge worldwide (FAO, 2014). In Africa, the dog population is unknown. However, cross-sectional household surveys have been used to determine the human: dog ratio, which estimates the dog population (Conan et al., 2015; Sambo et al., 2018). Across most of Africa, the estimated human: dog ratio is 8:1 in rural areas and higher in urban areas (Munyua et al., 2016). In 2015, Kenya had an estimated dog population of 6 million. Worldwide stray dogs are the key vector of the rabies virus, resulting

in tens of thousands of deaths annually. Ninety-nine per cent of these fatalities occur in the global south (Knobel et al., 2005). Rabies kills about 2000 people annually in Kenya (Munyua et al., 2016). About 4.2 million dogs need to be vaccinated continuously to meet the 70% target for elimination (Munyua et al., 2016). The aim of controlling or preventing reproduction in dogs is to reduce the population of unwanted animals humanely (FAO, 2014).

Population control is the exercise of artificially sustaining the size of any population. It simply refers to actively limiting the size of an animal population so that it remains manageable, as opposed to the act of protecting a species from excessive rates of extinction, which is referred to as conservation biology. Contraception is a globally accepted means of population control (Mathew and Bantwal, 2012). Across the species, various methods are used to control reproduction. These methods are helpful in population control among domestic and wild animals and Planned Parenthood in humans. Extensive studies into the events of folliculogenesis, ovulation, maintenance of the corpus luteum, and implantation using animal models have helped identify possible ways for contraceptive intervention and assisted reproductive technologies (Liechty et al., 2015). To achieve a contraceptive effect with negligible side effects, the expression and function of these agents should be exclusive to the reproductive tract or pathway. Events near fertilisation are targeted to avoid disrupting gametogenesis (Liechty et al., 2015).

A range of methods can be used, including surgical sterilisation, chemical or immunological sterilisation, contraception, and confinement during oestrus. There is increasing interest in reversible contraception, particularly where temporary or non-surgical approaches to fertility control are preferred (Asa, 2018).

2.7.1 SURGICAL AND CHEMICAL STERILISATION

Most surgical procedures involve general anaesthesia and multimodal analgesia. Some surgical methods used to sterilise animals include castration and vasectomy in males and ovariohysterectomy and ovariectomy in females. Castration involves the removal of testicles, while vasectomy consists of the cutting or tying off of the vas deferens. Both procedures may cause inflammation postoperatively in adult males, although such reactions are reduced in young animals. Animals undergoing vasectomy will retain sexual behaviour after because testosterone production in the testicles is unaffected. While vasectomy may be helpful in the preparation of teaser bulls, it may not be ideal for male dogs as they will continue to roam the streets to mate with bitches on heat, territory marks, and fight with other males.

Chemical castration has been used as a non-surgical method of sterilising male dogs. The process involves injecting a chemical into the testis, epididymis or vas deferens. This chemical causes sterility by inducing azoospermia over time (Fagundes et al., 2014). The advantages of chemical castration are that it requires less technical know-how than surgical methods; they are relatively inexpensive and can be used for mass sterilisation programmes. Some products currently in use include zinc gluconate compounds (Neutersol and Esterilsol) in South America (Liu, 2011). Zinc gluconate induces atrophy of the seminiferous tubules and impairs spermatogenesis (Brito et al., 2011; Fagundes et al., 2014). It, however, does not lower testosterone levels; hence the male secondary sex characteristics are retained (Oliveira et al., 2012). This can be advantageous or a disadvantage for overly aggressive dogs where castration is recommended for behaviour modification.

In females, ovariohysterectomy involves removing the ovaries and uterus, making it a better sterilisation method than ovariectomy. In addition to the anaesthetic risk, Ovariohysterectomy has been associated with adverse side effects such as weight gain (Allaway et al., 2017) and urinary incontinence (Pegram et al., 2019; Stöcklin-Gautschi et al., 2001). However,

ovariectomy, which involves the removal of the ovaries, is preferred due to its small surgical incision. The technique is, however, popular in the western world. Tying off or cutting the fallopian tubes or oviducts (tubectomy) has been attempted. However, it is not recommended as the female will still be under the influence of ovarian hormones and, therefore, show sexual behaviour and be at risk of pyometra.

2.7.2 HORMONAL AND IMMUNOLOGICAL CONTRACEPTION/STERILISATION

Some chemicals are available in the market for the contraception/sterilisation of dogs.

Oestrogens have been used for the control of reproduction in bitches. The mode of action is to prevent a luteinising hormone (LH) surge and interfere with ovulation. Diethylstilbesterol, an oestrogen, was previously used to rectify mismating in dogs. However, oestrogens have been associated with many side effects, including bone marrow suppression and aplastic anaemia (Asa, 2018). Synthetic oestrogens are no longer recommended for contraception due to these safety issues. However, it was recently established that oestradiol benzoate could be used as a contraceptive in dogs when administered a single dose (Tsutsui et al., 2006). There is no history of using oestrogens in cats due to safety and efficacy concerns.

The use of progestogens has been attempted for several decades. The most commonly used synthetic progestogens are megestrol acetate, medroxyprogesterone acetate, and proligestone. Progestogens work by causing a negative feedback effect on the hypothalamus suppressing the production of FSH, LH, and ovulation. In addition, they impede the movement of the ovum and spermatozoa to the ampulla and interfere with implantation. The use of progestins in dogs has been associated with several side effects, such as increased cases of cystic endometrial hyperplasia, mammary gland tumours, diabetes Mellitus, acromegaly, and immunosuppression. Incidences of cystic endometrial hyperplasia increase when the hormone

is administered during proestrus/oestrus because the uterus is primed with oestrogen. The use of progestins is therefore limited to dioestrus.

Androgens have been used for contraception in female dogs due to their negative feedback loop preventing LH surge and ovulation. However, their use in males has not been as successful as in females. In males, androgens suppress LH production and eventually interfere with spermatogenesis. However, they also stimulate libido and prostatic development in the male, which is undesired. The most commonly used androgen is mibolerone. It is used as an oral contraceptive administered daily and has been shown to work for up to 5 years. Some side effects encountered with androgens in females include clitoral hypertrophy, vaginitis, vaginal discharge, and masculine behaviours of mounting and aggression. Mibolerone is not approved for use in cats as it can cause fatal liver toxicity (Asa, 2018).

The gonadotropin-releasing hormone triggers the production of FSH and LH from the anterior pituitary, which then travels to the gonads¯ stimulating spermatogenesis and folliculogenesis in the male and female. GnRH agonists are used as contraceptives because continuous administration of GnRH causes downregulation of the HPG axis, eventually interfering with ovulation and spermatogenesis. Some of the GnRH agonists used in the market include deslorelin implant (Supralorelin®-Virbac) in European Union, Australia, and New Zealand (Lucas, 2014; Rhodes, 2017) and Azagly- nafarelin (Gonazon®: Intervet) (Hart et al., 2020). Supralorelin® is used mainly in male dogs and offers temporary sterility for about 6-12 months. Azagly- nafarelin was developed for use in female dogs; it did not reach market production (Rhodes, 2017). The main disadvantage of GnRH agonists as contraceptives is that they cause stimulation of oestrus and ovulation when initially administered. This initial flare means the females must be separated from the males for three weeks to prevent accidental mating. The use of progestins combined with the GnRH agonists has been suggested to control this flare. However, studies on greyhounds found the combination not useful (Asa, 2018). The

contraceptive does not affect the spermatozoa already within the genital tract in males. Hence, they must be kept separate from the females for up to 3 weeks.

The past two decades have seen a rising interest in using immunocontraceptives to control animal populations. Immunocontraception involves active vaccination against an antigen vital to reproductive function, with the resulting immune reaction reducing fertility. The main target area of immunocontraceptives is to impair gamete production, outcome, and function (Kaur and Prabha, 2014). Some of the agents targeted include gonadotropins which target gamete production (Faulkner et al., 1975) or zona pellucida proteins targeting gamete outcome (Mahi-Brown et al., 1982), and sperm antigens targeting gamete function (Kaur and Prabha, 2014). The porcine zona pellucida vaccine has been used successfully in wild horses. The vaccine prevents spermatozoa from penetrating the zona pellucida and fertilising the ovum. Another vaccine against gonadotropin-releasing hormone has also been used successfully in wild ungulates. No Immunocontraceptives have been developed successfully or used consistently in dogs or cats (Rhodes, 2017).

There are also barrier methods of contraception in dogs. Intrauterine devices have been designed to prevent pregnancy in dogs (www.dogspiral.vet). So far, they have shown minimal side effects; however, about 10% of the females using the devices presented with persistent oestrus. The mode of action for these devices is to stimulate a local inflammatory response that will interfere with implantation. However, the same foreign body reaction may lead to uterine pathology.

While these available products are practical and valuable under some circumstances, there is a need for an ideal nonsurgical agent for sterilisation in male and female dogs and cats. These products are unsuitable since they are limited to males, require booster injections, and are

effective for only a short time (Liu, 2011). Despite their limitations, these efforts are commendable due to their vision and missionary accomplishments.

2.8 PROSTAGLANDINS AND THEIR ROLE IN REPRODUCTION

Prostaglandins are lipid mediators produced from arachidonic acid. There are five types of prostaglandins produced by mammalian tissues: prostaglandin E2 (PGE2), prostaglandin D2 (PGD2), prostaglandin PGF2α (PGF2 Alfa), prostaglandin PGI2 (PGI2), and thromboxane (THA2) (Niringiyumukiza et al., 2018).

The biosynthesis of PGs can be divided into three phases: (a) release of the arachidonic acid from membrane phospholipids by the action of phospholipase A2 enzymes; (b) conversion of arachidonic acid into an unstable prostaglandin intermediate, PGH2, by cyclooxygenase enzymes; and (c) conversion of PGH2 into various biologically active prostanoid by cell-specific terminal synthases (Sirois et al., 2004). Prostaglandin, thromboxane and leukotrienes are collectively known as eicosanoids. Eicosanoids are synthesised from arachidonic acid, a 20-carbon fatty acid. Arachidonic acid is derived from the dietary intake of linoleic acid. Arachidonic acid is stored within the cell membranes attached to phospholipids. Following a trigger by either hormone, chemical or physical stimulus, Phospholipase A2 cleaves arachidonic acid from the cell membrane to initiate the synthesis of prostaglandins. The free arachidonate is then acted upon by the cyclo-oxygenase enzyme (COX), which inserts oxygen into the arachidonate to form prostaglandin G2. Tissue peroxidases then convert prostaglandin G2 to various prostaglandins (PGI, PGE2, PGF2) and thromboxane (TXA).

There are two cyclooxygenase isoenzymes, COX 1 and COX 2 (Simmons et al., 2004). COX 1 is found in most tissues of the gastrointestinal tract, kidney and Platelets (Gudis and Sakamoto, 2005). COX 2 is inducible and present in specific tissues following immune activation or inflammatory mediators (Rouzer and Marnett, 2009). COX 2 is responsible for the pain, swelling, heat and redness in inflammation and fever in infections. COX-1 is known to play a physiological role in the renal, cardiovascular and reproductive systems, while COX-2 is involved in ovulation, fertilisation, implantation, and maintenance of pregnancy (Gudis

and Sakamoto, 2005; Lim et al., 1997a; Rouzer and Marnett, 2009; Shah and Catt, 2005). However, the individual contributions of COX-1 or COX-2 to neuroendocrine reproductive function remain unknown.

2.8.1 PROSTAGLANDIN SIGNALLING PATHWAY

The prostaglandins have a relatively short half-life. They are local mediator molecules synthesised by cells and act within and around these cells in an autocrine or paracrine manner (Pettipher, 1998). They exert their effects by activating rhodopsin-like G protein-coupled receptors (GPCRs). There are eight members of the prostanoid receptor subfamily: E prostanoid receptor 1 (EP1), EP2, EP3, and EP4 subtypes of the PGE receptor, PGD receptor (DP1), PGF receptor (FP), PGI receptor (IP), and TX receptor (TP) (Narumiya & FitzGerald, 2001).

EP1 signaling is associated with the phospholipase C/inositol triphosphate pathway and intracellular calcium elevation; EP2 and EP4 activity are associated with increased cyclic AMP (cAMP); EP3 is thought to be inhibitory, and its activity is associated with decreased cAMP levels; and EP1 signaling is associated with the phospholipase C/inositol triphosphate pathway and intracellular calcium elevation (Wolf, 2011). EP2 and EP4 receptors also activate phosphoinositide 3-kinase (PI3K) through the -arrestin pathway (Sugimoto et al., 2014). Among the identified downstream targets of EP activity are the Mitogen-Activated Protein Kinase (MAPK), AKT, and PI3K/-catenin pathways (Krysan et al., 2005). Each PG has a unique mechanism and effect on the female reproductive system.

PGI causes smooth muscle relaxation and vasodilation and prevents the activation of platelets. PGE2 also causes smooth muscle relaxation and vasodilation {Citation}. PGI and PGE are formed from COX 1 and help increase blood supply to the affected organ or region. COX 2 is responsible for the production of PGF2 and TXA . COX 2 production is influenced by

cytokines, growth factors, endotoxins, and tumour factors. TXA causes platelet activation, and TXA and PGF2 cause smooth muscle contraction and vasoconstriction .

2.8.2 PROSTAGLANDINS IN REPRODUCTION

Prostaglandins (PGs) affect ovulation, luteal regression, the implantation and maintenance of pregnancy, parturition, and postpartum physiology (Weems et al., 2004). In livestock, prostaglandins are used to synchronise oestrus alone or combined with progestins, oestrogens, and gonadotropin-releasing hormone (Weems et al., 2004).

PGE2 is mammals' most common and substantial prostanoid (Niringiyumukiza et al., 2018). A wide range of receptor subtypes are bound by PGE2 and have multiple signal transduction properties. Experiments with PGE2 receptor type2 (EP2)-knockout mice showed disorders in ovulation, fertilisation, embryo development, and implantation (Chakraborty et al., 1996; Matsumoto et al., 2002). PGE2 stimulates the release of a luteinising hormone-releasing hormone (LHRH) in vivo (Ojeda et al., 1978) but does not directly influence the release of LH from the pituitary (Harms et al., 1973; Ojeda et al., 1978). Additionally, PGE2 may be involved in modulating the LHRH pulse generator (Weems et al., 2004). Park et al. (2004) demonstrated that PGE2 might affect the maturation of the LHRH neuronal system during puberty and the pulsatile secretion of LHRH.

Elevated levels of PGE are necessary for successful ovulation. In monkeys, elevated PGE2 late in the periovulatory interval increases the tissue plasminogen activator (tPA) protein via EP2 and EP3 while enhancing the PAI-1 protein via EP1 and EP3 in granulosa cells, which acts to stimulate proteolysis and follicle rupture (Markosyan and Duffy, 2009). PGE2 is involved in cumulus expansion and meiotic maturation (Yamashita et al., 2011) by increasing Cyclic adenosine monophosphate (cAMP) (a well-known mediator of meiotic development) production in follicles, resulting in the maturation and cumulus expansion of oocytes

(Yamashita et al., 2011). The PGE2 receptors EP2 and EP4, predominant in cumulus and granulosa cells, can increase intracellular cAMP levels when coupled to adenylate cyclase. Mice oocytes treated in vitro with selective EP2 and EP4 agonists increased cAMP production and ovulation rates (Duffy et al., 2010; Niringiyumukiza et al., 2018). PGE2 is synthesised in large quantities within the follicle. It is an essential mediator in the ovulation-stimulating action of gonadotropin. Upon gonadotropin stimulation, there is induction of COX-2 production in all the cells within the follicle; this is followed by the release of a large amount of PGE2 into the follicular fluid. COX-2-/- null mice show severely impaired ovulation (Lim et al., 1997b). Equally, mice lacking the PGE receptor EP2, expressed in the cumulus cells, exhibit reduced ovulation (Hizaki et al., 1999). These results show that PGE2-EP2 signalling in the cumulus plays a role in ovulatory processes.

PGF2α is synthesised from PGH2 via PGF synthase, and it acts via the FP, which couples with Gq protein to elevate the intracellular free calcium concentration (Ricciotti and Fitzgerald, 2011). Two differentially spliced variants of the FP receptor ortholog have been reported in sheep: FPA and FPB, which differ in the length of their C-terminal tails (Auletta and Flint, 1988). In the female reproductive tract, PGF2α is derived mainly from COX-1. It plays a vital role in ovulation, luteolysis, uterine smooth muscle contraction and initiation of parturition (Narumiya and FitzGerald, 2001; Sugimoto et al., 1997).

In the cycling female, CL development, maintenance, function, and regression are regulated by intricate interactions between luteotropic and luteolytic factors (Arosh et al., 2004). Prostaglandins are synthesised from the essential fatty acid arachidonic acid stored in membrane phospholipids (P. B. Smith et al., 1994). The corpus luteum cells and tissues in the reproductive system are a rich source of arachidonic acid and can produce prostaglandins (Olofsson and Leung, 1994). Prostaglandin E2 and F2α play critical roles in CL function and reproduction in general. Prostaglandin E2 plays several roles in different mammalian systems:

mitogenesis, angiogenesis, anti-apoptosis, and vasodilation (Arosh et al., 2004), and in the female reproductive system, it is luteotrophic while PGF2α causes luteolysis.

ProstaglandinF2α is the luteolytic agent in most mammals (Arosh et al., 2004). The luteolytic properties of PGF2 have been discussed under neuroendocrine control of the oestrous cycle.

Using reporter and mutant mice, Matsumoto et al. (2002) demonstrated the importance of COX-2-derived prostaglandins in angiogenesis and uterine vascular permeability during implantation and decidualisation (Dey & Lim, 2006).

COX-1 and -2, cPGES, mPGES-1and -2, and prostacyclin synthase have been reported at the implantation site in humans, rats, and mice, indicating that prostaglandins play a vital role in embryo implantation (Dey et al., 2004; Salleh, 2014). PGE2 and PGF2α have been shown to play a role in the spacing of blastocysts, implantation and decidualisation (Dey & Lim, 2006). PGE and PGF2 cause contraction of the circular endometrial muscles, which then spaces the blastocysts. In mice, there is an increase in the levels of PGE2 from the 2cell stage embryo up to the blastocyst stage. During the peri-implantation period, PGE2 also plays a vital role in expressing EP2 and EP4 receptors in mice uterus. This expression increases cAMP levels at the time of implantation and decidualisation. EP4 works in concert with PGF2 to activate VEGF, increasing vascular permeability of the endometrium, implantation and decidualisation. In mice and likely humans, reduced prostaglandin production has been postulated to cause infertility.

Prostacyclin, on the other hand, has been shown to affect implantation and decidualisation (Cong et al., 2006). Prostacyclin (PGI2) is the primary prostaglandin at the implantation site of mice. It influences implantation and decidualisation through the peroxisome proliferator-activated receptor (PPAR-d) and the RXRa signalling pathway in the uterus (Lim et al., 1999;

Mayoral Andrade et al., 2020). Low concentrations of prostaglandins E2, F and I2 have been shown to cause ovulation, fertilisation, implantation and decidualisation failure.

2.9 THE ENDOCANNABINOID SYSTEM IN REPRODUCTION

Endocannabinoids are lipid mediators that can moderate a range of biological processes, including mammalian reproduction (Ezechukwu et al., 2020). The discovery of the endocannabinoid system is attributed to continued research on cannabis. Cannabis is a natural plant extract with medicinal properties documented in both animal and human medicine (Russo et al., 2008). Cannabis contains numerous molecules, including over 60 chemical compounds classified as cannabinoids (Andre et al., 2016). The different Cannabis chemotypes vary in their cannabinoid composition (Almogi-Hazan and Or, 2020; Andre et al., 2016). There are over one hundred distinct cannabinoid extracts in cannabis. The two most comprehensively studied natural plant cannabinoids (phytocannabinoids) are Δ9 tetrahydrocannabinol (THC) and cannabidiol (CBD) (B. Park et al., 2004).

These cannabinoids interact with the endocannabinoid system causing the psychoactive effects of cannabis. Cannabis extracts like marijuana have a higher consumption rate worldwide than other recreational drugs (Cecconi et al., 2014). Their societal approval as recreational and therapeutic drugs severely threaten female reproduction because phytocannabinoids can interfere with an endogenous system of lipid signals known as endocannabinoids. Endocannabinoids are pharmacologically similar but differ structurally from phytocannabinoids (Cecconi et al., 2014). In summary, endocannabinoids are endogenous lipids that occupy cannabinoid receptors modifying behaviour in a manner that at best partially reiterates the effects produced by the psychoactive components of cannabis (Lu and Mackie, 2016).

Endocannabinoids (ECS) ligands, cannabinoid receptors type 1 (CB1) and 2 (CB2), and related enzymes are present in the hypothalamus-pituitary-ovarian axis (Cui et al., 2017; Fride, 2002). The first endocannabinoid identified in brain tissue was N-arachidonoylethanolamide (anandamide, AEA) (Devane et al., 1992); followed by arachidonoyl-glycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995), N-arachidonoyl-dopamine (NADA) (Bisogno et al., 2000), O-arachidonoyl- ethanolamine (Porter et al., 2002) and 2-arachidonoyl-glyceryl ether (2-AGE) (Petrocellis et al., 2004).

2.9.1 ANANDAMIDE SYNTHESIS TRANSPORT AND DEGRADATION

Anandamide (AEA) is the main endocannabinoid it was the first to be discovered and has been extensively studied compared to the others (di Marzo and Petrosino, 2007; Fezza et al., 2014). It is a lipophilic molecule that acts as a neurotransmitter. Anandamide is synthesised from precursors of phospholipids located in plasma membranes to a phosphatidylethanolamine, then the formation of N-acylphosphatidylethanolamine (NAPE). This is followed by the cleavage of a phospholipid precursor, N-arachidonoyl-phosphatidylethanolamine (NAPE), by a specific phospholipase D (NAPE-PLD) found in the inner layer of the cell membrane to produce AEA. AEA moves across the phospholipid bilayer by simple diffusion or endocytosis. However, there have been strong suggestions that this movement is aided by the supposed endocannabinoid membrane transporter (EMT), which enables rapid transport of anandamide to its various intracellular target (Cecconi et al., 2014; Chicca et al., 2012; Oláh et al., 2017). Unlike conventional neurotransmitters that are synthesised and stored within synaptic vesicles for release upon stimulation, endocannabinoid precursors are present in lipid membranes and are liberated on demand in one or two enzymatic steps and released into the extracellular space (Lu and Mackie, 2016).

Anandamide intracellular binding proteins (AIBPs), specifically albumin, fatty acid-binding protein-5 and -7 (FABP-5 and -7) and heat shock protein 70 (Hsp70), have been identified as

intracellular targets for AEA (Brents, 2016; Chicca et al., 2012; Walker et al., 2019). Intracellular hydrolases convert anandamide into ethanolamine and arachidonic acid. FAAH is bound to intracellular membranes, including endoplasmic reticulum and nuclear membranes, and regulates anandamide activity (Walker et al., 2019).

Anandamide can diffuse freely through cell membranes but, unlike most neurotransmitters, is not stored in vesicles (Fegley et al., 2004; Maia et al., 2017). Therefore, its local concentration is closely controlled by balancing its on-demand synthesis and degradation (Maia et al., 2017).

Cannabinoids exert their action through cannabinoid receptors 1 and 2. Both CB1 and CB2 belong to the G protein-coupled receptors superfamily (Walker et al., 2019). CB1 is located within the central nervous system and peripherally in the adrenal glands, heart, ovaries, spleen, endometrium and testes (Walker et al., 2019). It is also located intracellularly on the outer membrane of mitochondria (Walker et al., 2019). CB1 receptors are found in the hypothalamus, which is the central regulator of energy homeostasis, suggesting that the endocannabinoid system is involved in energy balance (Walker et al., 2019). CB1 receptors are also present in the preoptic region of the hypothalamus, where secretory neurons for gonadotropin-releasing hormone (GnRH) are located (Walker et al., 2019).

CB2 receptors are predominantly located on the periphery of immune system cells, such as macrophages and lymphocytes (Walker et al., 2019). Additionally, CB2 receptors have been identified in the ovarian medulla, cortex, and follicles of human samples (El-Talatini et al., 2009; Walker et al., 2019). In contrast, CB1 receptors mediate endocannabinoid action on murine oocytes as opposed to CB2 receptors in humans (Wang et al., 2008).

In addition to CB1 and CB2 receptors, endocannabinoids also target other receptors, including GPR55 (also known as CB3) and GPR119 receptors, which have signalling mechanisms distinct from CB1/CB2. Other receptors targeted by endocannabinoids include TRPV1,

cytosolic target for AEA, and nuclear PPAR. A detailed review of these other receptors and their pharmacology is published (Pertwee and Ross, 2002).

2.9.2 ENDOCANNABINOIDS IN REPRODUCTION

The accumulated evidence from human and animal models indicates that these endogenous signals play a crucial role in various phases of female reproduction (Walker et al., 2019). They exert their effects by means of proteins that synthesize, transport, degrade, and transport them. Recent studies have demonstrated the potential role of endocannabinoids as biomarkers of female infertility in the treatment and prevention of disease, as well as their possible epigenetic effects on pregnancy (Walker et al., 2019).

CB1 receptors have been identified in the hypothalamus and anterior pituitary, whereas CB1/CB2 receptors have been identified in the ovary (Cecconi et al., 2014; El-Talatini et al., 2009; Gammon et al., 2005; Walker et al., 2019). In addition, ECS components such as AEA and FAAH have been detected in ovaries, oviducts, endometrium, and myometrium (El-Talatini et al., 2009), indicating that they are involved in reproduction. As a result of the observation that cannabis derivatives alter the reproductive process, researchers are examining the effects of endogenous cannabinoids on the HPG axis. This effect is observed across species and gender. These modifications include a decrease in circulating GnRH, an anovulatory cycle, a prolonged follicular phase, and a delay in ovulation (Brents, 2016). The majority of reports suggest that these effects result from hypothalamic dysfunction (Walker et al., 2019). Others have hypothesized that the effect may be mediated at the pituitary or ovarian level (Gammon et al., 2005; Brents, 2016; Walker et al., 2019), although the precise mechanism is unknown (Gammon et al., 2005; Walker et al., 2019). Moreover, the presence of CB1 on the outer mitochondrial membrane (Bénard et al., 2012; Walker et al., 2019) and the relationship between mitochondrial function and oocyte (May-Panloup et al., 2016; Walker et al., 2019) are detrimental to the disruption of the endogenous cannabinoid-dependent regulation of oogenesis

by THC or other cannabinoids (Walker et al., 2019). It suggests that it may have effects on fertility. Currently, direct mechanical support for this hypothesis is absent (Walker et al., 2019).

Cannabinoid receptors CB1 and CB2 have been localized in all stages of oocyte development in humans (El-Talatini et al., 2009). In contrast, the enzymes NAPE-PLD and FAAH have only been identified in the expanding corpora lutea and Albicans follicles (El Talatini et al., 2009). This data indicates that the ovary is capable of producing AEA, which may play a role in folliculogenesis, preovulatory follicle maturation, oocyte maturation, and ovulation (El-Talatini et al., 2009).

It is hypothesized that endocannabinoid signaling helps regulate follicular development and maturation. During the ovarian cycle, AEA levels fluctuate, with an increase in the ovary just prior to ovulation (El-Talatini et al., 2009). At the time of ovulation, high levels of anandamide are required within the follicles. Nonetheless, excessively high levels of anandamide inhibit folliculogenesis and ovulation by acting on the hypothalamus and granululosa cells (Walker et al., 2019). According to in vitro studies, THC inhibits folliculogenesis and ovulation directly in rat granulosa cells. Ovulation requires the accumulation of cAMP, which THC inhibits. Adashi et al. (1983) discovered that THC or its metabolites inhibited the FSH-stimulated accumulation of progesterone and oestrogen as well as the FSH-stimulated increase in LH receptors. It was determined that this direct effect of THC occurs after the formation of cAMP and involves the inhibition of steroidogenesis (Walker et al., 2019). THC inhibits the conversion of pregnenolone into progesterone. To corroborate these findings, it has been demonstrated that occasional and moderately heavy cannabis users exhibit anovulatory cycles leading to primary infertility (Brents, 2016a; Cecconi et al., 2014).

In males, chronic exposure to cannabinoids has been associated with oligospermia and lowered testosterone and LH levels (Gammon et al., 2005). Similarly, in females, exposure to

cannabinoids has been shown to cause a delay in sexual maturity, disrupt the menstrual cycle in humans, disrupt the maturation of follicles, and lower LH and sex steroid hormone concentrations in serum (Gammon et al., 2005).

Preimplantation mice embryos express both CB1 and CB2 receptors. However, only CB1 is expressed in the oviduct and uterus (Maccarrone, 2008), correlating to a high affinity for anandamide. CB1 mRNA expression can be identified from the 4-cell stage embryo to the blastocyst, while CB2 is found from the 1-cell stage through to the blastocyst (Paria et al., 1995). These receptors in the blastocyst suggest that mice embryos are targets for both natural cannabinoids and endocannabinoids (Wang et al., 2006). Indeed, 2-cell embryos fail to develop into blastocysts when exposed to natural (THC), synthetic (WIN 55212-2, CP 55940), or endocannabinoids (AEA and 2-AG) in vitro (Paria et al., 1995; Yang et al., 1996). CB1 − / −, CB2 − / −, or CB1 − / − x CB2 − / − knockout mice embryos recovered on the third and fourth day from the oviduct and uterus showed asynchronous development compared to wild-type embryos(Paria, et al., 2001; Wang et al., 2004). This aberrant embryo development is reversed by breeding CB mutant females to wild-type male mice. This produces heterozygous embryos in a mutant environment indicating that embryonic cannabinoid receptors control the synchronous development. Regular embryo transport through the oviduct is one of the prerequisites for well-timed implantation. Therefore, dysfunctional regulation of this process results in oviductal embryo retention and may increase the incidence of pregnancy failure.

Anandamide and CB1 receptor expressions in the uterine tissue in mice are higher than in the brain tissue (Cui, et al., 2017; Yang et al., 1996). Anandamide plays a vital role in the synchronous development of the endometrium and embryo. Low anandamide levels are required at the implantation sites for successful implantation (Habayeb et al., 2008). Maccarrone (2009) demonstrated that high intrafollicular levels of AEA allow for ovulation, while low plasma and intrauterine levels are required to implant a fertilised oocyte.

AEA exerts its effects on implantation by binding to the CB1 receptors expressed in the uterus and blastocysts (Turco et al., 2008). High levels of anandamide in the female reproductive tract negatively affect the developing embryo, while low levels stimulate its growth (Cui, et al., 2017; Paria, et al., 2001; Yang et al., 1996). This increase is either a result of increased synthesis or decreased rate of degeneration (Maccarrone et al., 2000). These studies provide pharmacological, molecular, and genetic evidence that the preimplantation embryo is a target for cannabinoid/endocannabinoid signalling (Dey et al., 2004).

In addition to being present in placental tissues, the ECS plays a crucial role in placental development. Multiple studies utilizing knockout mouse models have demonstrated this (Maia et al., 2020). Compared to wild-type mice, trophoblast cells proliferate less in CB1/mice, and the placenta weighs less in CB1/mice (Sun and Dey, 2012). A decrease in trophoblast proliferation was also observed in FAAH/animals, indicating that ECS plays a role in trophoblast proliferation and differentiation (Sun and Dey, 2012).

Through its conversion to AM404, Acetaminophen may increase anandamide levels by inhibiting its uptake into the intracellular space for degradation. The resulting high levels of anandamide may then interfere with the developing embryo.

2.10 ACETAMINOPHEN

Acetaminophen (N-acetyl-p-aminophenol or Paracetamol) is readily available globally as an over-the-counter analgesic. Acetaminophen was first synthesised in 1893 by Joseph von Mering by reacting p-nitrophenol with glacial acetic acid and tin (Ayoub, 2021). Around 1880 phenacetin and paracetamol were found to possess antipyretic and analgesic activity. Clinical use of Phenacetin was discontinued as it was found to cause haemolytic anaemia, methaemoglobin formation and was carcinogenic (Brodie and Axelrod, 1949). It was also later discovered that paracetamol was one of the metabolites of phenacetin and that the

pharmacological effects of phenacetin were attributed to it. Around 1950, acetaminophen became the drug of choice for treating fever and pain. The use of acetaminophen has been on the rise since the 1960s, when aspirin was discovered to cause gastrointestinal toxicity and was associated with Reye syndrome in children (Ayoub, 2021). To date, the use of acetaminophen has been on a steady rise and is expected to remain the same due to its safety at therapeutic doses (Ayoub, 2021).

2.10.1 PHYSICOCHEMICAL PROPERTIES

Acetaminophen is an odorless, white, crystalline, bitter-tasting solid with a molecular weight of 151.16g/mol. It is slightly acidic (pKa 9.0–9.5) and unionises effectively at physiological pH levels. At 25°C, it has a pH range of 5.3 to 6.5 and a density of 1.293 g/cc. Its octanol/water partition coefficient (Kow) is 6.2, which falls within the range for passive diffusion across cell membranes. About 170°C is the melting point of acetaminophen. It has a low solubility in cold water, but a high solubility in hot water (14,000 mg/L at 25°C) (Yalkowsky et al., 2016). It is freely soluble in alcohol, ethanol, methanol, ethylene, dimethylformamide, acetone, dichloride, and ethyl acetate, and only slightly soluble in ether. However, it is essentially insoluble in pentane benzene and petroleum ether (Fadel et al., 2021).

Acetaminophen is chemically a phenol which is easily oxidised. There are three steps involved in the synthesis of acetaminophen (Gayflor-Kpanaku et al., 2013). Electrophilic aromatic substitution is first used to convert phenol to nitrophenol. This is followed by either sodium-borohydride ($NaBH_4$) reduction or direct hydrogenation of the nitro group of the para-substituted nitrophenol to an amine. The para-aminophenol is finally converted to APAP via a reaction with acetic anhydride.

Despite being used for almost a century, acetaminophen's exact mode of action is not fully understood. Acetaminophen acts as an antipyretic and analgesic but has weak anti-

inflammatory properties. Acetaminophen is a very poor cyclo-oxygenase inhibitor and does not inhibit neutrophil activation; hence it does not possess anti-inflammatory properties (Ohashi and Kohno, 2020). Acetaminophen is safe and usually prescribed to patients to whom non-steroid anti-inflammatory drugs are contraindicated.

2.10.2 ACETAMINOPHEN METABOLISM

In humans, acetaminophen is primarily administered orally, although there is an increase in postoperative intravenous administration. Oral administration results in a delayed onset of action compared to the intravenous route. Following oral administration, acetaminophen has a high bioavailability and is well absorbed within the duodenum, reaching peak blood concentrations within one and a half hours (Hodgman and Garrard, 2012; Mazaleuskaya et al., 2015). In dogs, acetaminophen is used as a postoperative analgesic at a 10-15mg/Kg dosage rate (Hernández-Avalos et al., 2020). The pharmacokinetics of intravenous acetaminophen at a dosage rate of 10 mg/kg and 20 mg/kg have been studied in beagles, Labradors and Spanish sighthounds (Serrano-Rodríguez et al., 2019). At this dose, acetaminophen did not affect the clinical biochemistry values of these dog breeds. However, the pharmacodynamics following intravenous administration of acetaminophen in dogs has not been studied (Leung et al., 2021).

After administration of a therapeutic dose, Acetaminophen undergoes conjugation with glucuronides and sulphates in the liver to form pharmacologically inactive conjugates which are excreted in urine (Salomone et al., 2017). There are three metabolic pathways involved in acetaminophen metabolism: Glucuronide conjugation, sulphate conjugation and cytochrome P450 pathway.

Acetaminophen glucuronidation is catalyzed by UDP-glucuronosyl transferases (UGT) (Bock *et al.*, 1993; Court, 2001). UGTs transfer the glucuronosyl group from uridine 5'-diphosphate-glucuronic acid (UDP-glucuronic acid) to increase the water solubility of the acetaminophen

molecule (Mazaleuskaya *et al.,* 2015). About sixty percent of administered acetaminophen is metabolized via glucuronidation. Four families of UGTs have been identified in rodents and humans: UGT1, UGT2, UGT3, and UGT8. Human in-vitro studies utilizing cultured hepatocytes and liver microsomes suggest that UGT1A1, UGT1A6, UGT1A9, and UGT2B15 participate in APAP glucuronidation. UGT1A6 is required at low APAP concentrations (Court *et al.,* 2001), whereas UGT1A9 and UGT1A1 contribute the most at toxic doses, with UGT1A9 catalyzing over a wide range of pharmacologically relevant APAP concentrations (Court *et al.,* 2001; Mutlib *et al.,* 2006). Genetic polymorphisms in UGTs have been shown to influence APAP metabolism in healthy individuals (Zhao and Pickering, 2011), as well as in disease states (de Morais *et al.,* 1992) and in response to a particular diet (Zhao and Pickering, 2011; Navarro *et al.,* 2011).

A family of cytosolic enzymes called sulfotransferases (SULT) sulfate acetaminophen. SULTs transfer a sulphonate group from the substrate 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to acetaminophen, thereby rendering it more polar and excretable (T. Liu *et al.,* 2010; Yamamoto *et al.,* 2015). Yamamoto et al. demonstrated that among the twelve human sulfotransferases, three (SULT1A1, SULT1A3, and SULT1C4) exhibited the highest sulphating activity towards acetaminophen (Yamamoto *et al.,* 2015). In the foetal liver, SULT1A3/C4 plays a significant role in APAP sulfation; however, in postnatal development, SULT1A1 and SULT2A1 sulfate APAP predominately, while SULT1A3/C4 activity decreases. Sulphation of acetaminophen occurs primarily in the liver and intestines (Yamamoto *et al.,* 2015). Approximately thirty percent of acetaminophen is metabolized via sulfation. Mice lacking the kidney transporter NaS1 have been shown to be more susceptible to APAP hepatotoxicity (Lee *et al.,* 2006). NaS1 is involved in the reabsorption of inorganic sulphate (SO42), and humans are known to exhibit NaS1 pleiomorphisms.

Approximately 5% of acetaminophen is metabolized into the toxic compound N-acetyl-p-benzoquinone imine (NAPQI) (Mazaleuskaya *et al.*, 2015), which is then conjugated with glutathione to form nontoxic cysteine and mercapturic metabolites (Hodgman and Garrard, 2012). The primary cytochrome p450 (CYP) enzyme responsible for the oxidation of acetaminophen to the reactive metabolite NAPQI is cytochrome 2E1. NAPQI is a pliable electrophile that readily reacts with nucleophilic sulfhydryl groups (Mcgill and Jaeschke, 2013). CYP2e1-deficient mice are resistant to APAP-induced liver damage (Lee *et al.*, 1996). Studies involving healthy human volunteers pre-treated with the CYP2E1 inhibitor disulfiram confirm that cyp2e1 is essential for the oxidation of acetaminophen (Lee *et al.*, 1996; Patten *et al.*, 1993).

2.10.3 EFFECT OF ACETAMINOPHEN ON PROSTAGLANDIN PRODUCTION

Acetaminophen competitively inhibits prostaglandin synthesis (Aronoff et al., 2006; H. S. Smith, 2009). Prostaglandins (PGs) are synthesised from arachidonic acid by the vital enzyme cyclooxygenase (COX), also known as prostaglandin synthase protein (PTGS). There are two distinct COX isoenzymes, COX-1 and COX-2. The two enzymes have different patterns of expression and regulation in mammalian cells. COX-1 appears to be constitutively expressed in most tissues and is responsible for the physiological production of PGs . COX-2 is induced by cytokines, mitogens, and endotoxins in inflammatory cells and is therefore responsible for the elevated production of PGs during inflammation .

Acetaminophen competes with arachidonic acid for the binding site of the Cyclo-oxygenase (COX) enzyme (Aronoff et al., 2006; Botting, 2000). COX oxidation is essential to exert its enzymatic activity (Sharma and Mehta, 2014). Acetaminophen acts as a reducing co-substrate at the prroxidase site, indirectly lowering the oxidised form of COX and interfering with this

activity (Sharma and Mehta, 2014). The peroxidase function of COX-1 and COX-2 may have two main effects on APAP metabolism.

To begin with, the metabolism uses reduced glutathione, and there may be local glutathione depletion. Reduced glutathione is a cofactor for various enzymes, including membrane-associated PGE synthase (Smith, 2009). As a result of the local shortage of reduced glutathione, PGE_2 synthesis may be inhibited. The second possible consequence of APAP metabolism is that the two reactive metabolites may combine directly with enzymes involved in PG synthesis and inhibit them (Graham and Scott, 2005; Smith, 2009). Acetaminophen is more effective in low peroxide tone and low arachidonic acid levels, such as those found in the CNS. Acetaminophen at low concentrations triggers prostaglandin synthesis and the reverse is true at high concentrations (Botting, 2000).

There have been suggestions of a third isoenzyme, COX-3, through which acetaminophen is thought to exert analgesia (Botting and Ayoub, 2005; Chandrasekharan et al., 2002). COX-3 is transcribed from the same gene as COX-1 (Chandrasekharan et al., 2002). In dogs, COX-3 has been identified in the cerebral cortex, with small amounts found peripherally (Botting and Ayoub, 2005; Chandrasekharan et al., 2002). COX-3 is, however, not found in humans (Schwab et al., 2003). Unlike COX-1, it produces functionally different polypeptides that are highly sensitive to analgesic/antipyretic drugs but exert low anti-inflammatory effects (Jóźwiak-Bebenista and Nowak, 2014). However, its activity and physiological effects in rodents, dogs, and humans have not been fully understood (Fadel et al., 2021; Kis et al., 2005).

The effect of Acetaminophen on COX will result in reduced prostaglandin production, and this will affect reproductive events by interfering with corpus luteum development (PGE inhibition) or preventing lysis (PGF2α) of the corpus luteum, consequently disrupt the oestrous cycle by prolonging the life span of the corpus luteum. By studying the length of the oestrous cycle in

treatment and control groups, this study will determine if acetaminophen affects the cycle, which will be attributed to its action on prostaglandins.

Acetaminophen has a dual action on prostaglandins: at low concentrations, it stimulates and at high concentrations inhibits the synthesis of PGs (Robak et al., 1978).

2.10.4 EFFECT OF ACETAMINOPHEN ON THE CANNABINOID SYSTEM

Acetaminophen undergoes deacetylation in the liver by the enzyme N-deacetylase to yield a minor metabolite, p-aminophenol (Högestätt et al., 2005; Mazaleuskaya et al., 2015). After deacetylation, In the central nervous system, acetaminophen is conjugated with arachidonic via Fatty Acid Amide Hydrolase (FAAH) to form N-arachidonoyl- phenolamine (AM404) (H. S. Smith, 2009; Walker et al., 2019; Zygmunt et al., 2000). AM404 is generally recognised as the most significant mediator of acetaminophen metabolite-induced analgesia. It is a potent transient receptor potential vanilloid type 1 (TRPV1) agonist (Walker et al., 2019; Zygmunt et al., 2000). Mallet et al. (2008) demonstrated that the inhibition of FAAH suppressed the antinociceptive effect of acetaminophen.

AM404 controls inflammation and oxidative stress. Its effect on reducing oxidative stress is associated with the existence of a phenolic group in its structure (Saliba et al., 2017; Saliba et al., 2018). In a murine model of inflammation, AM404 reduced the production of the cytokines interleukin (IL)-1β and IL-6 and amplified circulating tumour necrosis factor (TNF)-α levels (Saliba et al., 2017; Saliba et al., 2018). In a rat model of neuropathic pain, AM404 prevented the overproduction of nitric oxide (NO) and TNF-α and increased IL-10 production (Saliba et al., 2017; Saliba et al., 2018).

After nerve injury, cytokines, like tumour necrosis factor-α (TNFα) or interleukin-10 (IL-10), appear to be vital for the initiation of pain behaviour producing angiogenic (Perkins et al., 1995) and anti-hyper-algesic (Wagner et al., 1998) effects, respectively. Nitric oxide (NO) also

transmits and modifies nociceptive information at the periphery, spinal cord, and supra-spinal levels. Systemic administration of NO synthase (NOS) inhibitors may relieve chronic allodynia-like symptoms in rats with spinal cord injury (Hao and Xu, 1996).

N-arachidonoyl-phenolamine selectively binds to the CB1 receptor and inhibits anandamide's cellular uptake, increasing its extracellular space (Fegley et al., 2004; Högestätt et al., 2005; Ruggieri et al., 2008). Anandamide must be transported back into the intracellular space for degradation through either FAAH or COX (Maccarrone, Valensise, et al., 2000). Anandamide increase stimulates CB1 receptors via FAAH (Di Marzo and Deutsch, 1998).

This study utilised the mouse model because they have the highest levels of anandamide in the preimplantation uterus compared to other mammalian tissue (Schmid et al., 1997), with levels fluctuating through the various stages of pregnancy. This study seeks to demonstrate how Acetaminophen, through its action on the endocannabinoid system, may affect the implantation process by counting the number of implantation sites in treatment and control groups and studying the histology of the endometrium in preparation for implantation.

2.10.5 ACETAMINOPHEN TOXICITY

Acetaminophen can be found alone or combined with other molecules, making it versatile and prone to overdosing. Acetaminophen toxicity is attributed to overdose or excessive dosing (Mazaleuskaya et al., 2015). At supra-therapeutic doses, Acetaminophen sulfation becomes saturated with comparative increases in glucuronidations and, importantly, oxidation to NAPQI (Hodgman and Garrard, 2012). The continued production of NAPQI eventually depletes glutathione. At about 70% depletion of glutathione, NAPQI binds to cellular proteins resulting in cell injury (Hodgman and Garrard, 2012). However, several observations challenge this idea. First, the non-hepatotoxic meta isomer of APAP, 3’-hydroxyacetanilide (AMAP), binds to proteins despite having a less severe effect on hepatic glutathione (GSH) levels (Kenna,

2013; Rashed et al., 1990). Secondly, protein binding is detectable in human HepaRG cells within one hour of APAP treatment before GSH depletion (Mcgill et al., 2011). Finally, acetaminophen-protein adducts (APAP-CYS) can be measured in human serum after therapeutic doses (Heard et al., 2011). Despite this, there is a clear inverse relationship between APAP metabolic activation and GSH levels, and measuring the early hepatic GSH depletion kinetics remains one of the best ways to assess NAPQI formation (Mcgill and Jaeschke, 2013).

It has been demonstrated that NAPQI and GSH react enzymatically and spontaneously. Glutathione-S-transferases are enzymes that catalyze enzymatic GSH conjugation (GST). It was assumed that the enzymatic conjugation of APAP and GSH was most likely carried out by GST-Pi. The enzymatic reaction produces APAP-GSH and free APAP, while the spontaneous reaction produces 3-(glutathione-S-yl)-acetaminophen (APAP-GSH), a GSH conjugate, free APAP, and glutathione disulphide (GSSG), an oxidation product.

Detoxification of NAPQI occurs through its binding to the sulfhydryl group of glutathione (GSH) to form APAP-GSH, which is eventually expelled in urine as cysteine and mercapturic acid conjugates (Bessems and Vermeulen, 2001; Ketterer et al., 1983; Mazaleuskaya et al., 2015). After administration of 500mg/kg of acetaminophen in dogs, only 1% is excreted as APAP-mercapturic acid (Fadel et al., 2021).

N-acetylcysteine (NAC) is the recommended antidote for acetaminophen toxicity in dogs and cats and liver necrosis in men (Fadel et al., 2021). NAC is a precursor of GSH. By directly interacting with NAPQI to create an acetyl-cysteine conjugate that is eliminated in bile, it aids in restoring GSH levels. Additionally, by delivering extra amino acids and utilising them as energy substrates, NAC replenishes hepatic ATP levels and supplies mitochondrial energy substrates in the Krebs cycle (Lauterburg and Mitchell, 1982; Saito *et al.*, 2010). It has been shown that cimetidine, an inhibitor of certain cytochrome oxidase enzymes, reduces the

synthesis of NAPQI by blocking CYP 450. (Peterson *et al.*, 1983). Animals like dogs that get centrilobular necrosis as a result of NAPQI would benefit from this (Mitchell, 1977; Peterson *et al.*, 1983; Sajedianfard *et al.*, 2009).

Several natural products and herbal therapeutics, such as *Gentiana Manchuria* Kitagawa (GM), are thought to protect against APAP hepatotoxicity (Eugenio-Pérez et al., 2016; Zhao et al., 2012). This is achieved through their action as antioxidants or cell death signalling disruptors. However, there is debate whether they prevent hepatotoxicity by interfering with APAP metabolic activation (Jaeschke et al., 2010 and 2012).

Acetaminophen's poisonous effects are extremely dangerous for cats at any dosage. Less isoforms of the glucuronyl transferases enzymes are present in cats, which makes it easier for acetaminophen to be conjugated to glucuronic acid (Court, 2013). The majority of the medication is converted to sulphates as a result of the relative insufficiency of the glucuronide conjugation pathway, while the capacity of the sulfation pathway is lower in cats than in other species due to the relative deficiency of the glucuronide conjugation pathway. Acetaminophen stays in the circulation after the sulfation pathway reaches capacity, and more of it is converted to NAPQI by cytochrome P450 enzymes. In the presence of NAPQI, glutathione stores are rapidly depleted, and high acetaminophen doses inhibit glutathione synthesis (Lauterburg and Mitchell, 1982). (Walker *et al.*, 2016). In cats, NAPQI mostly affects the erythrocytes. The sulfhydryl groups of the globulin chains are vulnerable to oxidative damage because it initially binds to iron in heme. Due to its electrophilic nature, it also causes the oxidation of ferrous iron (Fe2+) to ferric iron (Fe3+), which changes hemoglobin into methaemoglobin (Allen, 2003). Haemoglobin oxidation causes it to become denaturated and precipitate onto the surface of erythrocytes, where it manifests as small granules called Heinz bodies (Allen 2003). Haemolytic anemia is caused by the erythrocytes becoming more and more brittle when Heinz bodies form. Methaemoglobin production in cats causes inadequate oxygen transport, which is

shown in the affected animals' respiratory discomfort, sadness, and frailty. Jaundice, haemoglobinuria, and mucous membranes that are dark brown or pale are symptoms of hemolytic anemia. The enzymes N-acetyltransferase (NAT) 2 and thiopurine methyltransferase are also lacking in cats (TMPT). Cats are more likely to develop methemoglobinemia than acetaminophen-induced hepatotoxicity, which may be explained by NAT2 deficiency (Court, 2013).

In young mice without any underlying risk factors, daily low-dose administration of acetaminophen does not cause hepatotoxicity (de Meijer et al., 2013; Hodgman and Garrard, 2012; Kondo et al., 2012; Yisarakun et al., 2014). Pre-treatment with moderate dose acetaminophen was found to either protect against (Ghanem et al., 2009; O'Brien et al., 2000; ShayiqA et al., 1999) or enhance the risk of toxicity (Kim et al., 2009) from further acetaminophen doses. Acetaminophen is widely accepted for use due to its safety at therapeutic doses. This is so because other non-steroidal anti-inflammatory drugs (NSAIDs) have been associated with side effects of nephrotoxicity and gastrointestinal bleeding.

There are several pathways through which Acetaminophen exerts analgesia: inhibition of prostaglandin synthesis or stimulation of nitric oxide (NO), serotonergic, opioid, and cannabinoid pathways (Sharma and Mehta, 2014). This study will review the effect of Acetaminophen on prostaglandin synthesis and activation of the cannabinoid system.

CHAPTER 3

3.0 GENERAL MATERIALS AND METHODS

3.1 SAMPLE SIZE DETERMINATION

Information on the effect of Acetaminophen on implantation sites in mice is lacking; hence in the calculation of sample size, it was assumed that the average number of implantation sites would reduce from 10 to 8 in the control and treatment groups respectively, with a standard deviation of 1 for both groups. Based on these, it was determined that four mice would be needed per group based on a 95% confidence interval and 80% power (two-tailed). One female mouse was added to each group to take care of any unexpected losses; hence, each study group had five mice.

3.2 CARE AND WELFARE OF EXPERIMENTAL ANIMALS

Experimental procedures and all ethical considerations on the use of animals were carried out according to the Kenyan animal welfare guidelines, Ethical approval was obtained from the Faculty of the Veterinary Medicine University of Nairobi Biosafety, Animal Use, and Ethics committee – FVM BAUEC/2019/187 (Appendix 1).

Female Swiss white mice were used for the study. They were aged between 8 and 10 weeks old and weighed between 20 and 35g. The animals were procured from the Kenya Medical Research Institute (KEMRI). They were housed in cages within the laboratory in the Department of Veterinary Pathology, Microbiology and Parasitology of the University of Nairobi. The mice were caged in groups of five and maintained under approximately 12-hour photoperiod and room temperature conditions. The mice had ad *libitum* access to water and a commercially obtained diet of mice pellets (Unga Limited®). All the mice were subjected to an initial acclimatisation period of two weeks before the start of the experiments.

The mice were monitored daily for the first ten days for health, weight, and cyclicity through specific cytological features that distinguish the four stages of the oestrous cycle -Dioestrus, Proestrus, Oestrus, and Metestrus (Cora et al., 2015). Only those with regular 4–5-day oestrous cycles were used for the study. Proven fertile male mice were housed with the females but in separate cages.

The study design was completely randomised; one group of animals received treatment, and the other group was a negative control that received physiological saline. Using a coin toss, the mice were randomly allocated into treatment and control groups. The mice were humanely sacrificed using pressurised carbon dioxide gas at the appropriate time.

3.3 STATISTICAL ANALYSIS

Data were entered in Microsoft excel and then transferred to SPSS version 12.0 for windows (SPSS, Chicago, Illinois, USA) for analysis. Student t-test was used to examine the difference between groups and ANOVA was used to compare the means after three runs of the experiments. P-value ≤ 0.05 was considered statistically significant.

CHAPTER 4

4.0 EFFECT OF ACETAMINOPHEN ON THE OESTROUS CYCLE AND HORMONE PRODUCTION OF FEMALE MICE

4.1 INTRODUCTION

The oestrous cycle refers to the cyclical pattern of ovarian activity that allows a female mammal to transition from a phase of reproductive receptivity to a period of non-receptivity, with the ultimate goal of producing a pregnancy after mating (Crowe, 2020). The cycle consists of two phases: the follicular and luteal phases. The follicular phase concludes with ovulation and involves the recruitment, selection, and maturation of follicles. The corpus luteum is formed during the luteal phase by luteinisation of granulosa and theca cells. Once a mature tertiary follicle undergoes ovulation, it transforms into a corpus haemorrhagicum, followed by a progesterone-producing corpus luteum. A disruption of folliculogenesis will result in either a delay in ovulation or anovulation. Each phase of the oestrous cycle in mice is characterized by unique cell types that may be detected using direct microscopy (Folorunsho Ajayi and Eghoghosoa Akhigbe, 2020).

Hormones produced by the hypothalamus, pituitary gland, and ovary (hypothalamo- pituitary-gonadal axis (HPG) regulate the oestrous cycle in mammals (Klein, 2003). Gonadotropin Releasing hormone (GnRH) is released from the hypothalamus into the hypophyseal-portal system; from here, it travels to the anterior pituitary, where it triggers gonadotrophs to release luteinising hormone (LH) and follicle-stimulating hormone (FSH) into the circulation. These are then transported to the testes and ovaries, where they stimulate the production and release of sex steroid hormones, testosterone, oestrogen (E2), and progesterone. These sex hormones are then transported back to the hypothalamus through the circulation to provide positive or negative feedback, completing the HPG axis (McIlwraith and Belsham, 2020).

The regulation of the oestrous cycle is mediated by prostaglandins (PGs). Reproductive failures may be related to the inhibition of prostaglandin production. It has been demonstrated that nonsteroidal anti-inflammatory medicines (NSAIDs) influence the female reproductive system, resulting in reversible infertility (Stone *et al.,* 2002). However, because acetaminophen is not a typical NSAID, very little research has been conducted to determine its influence on the oestrous cycle. A disturbance at any stage of the HPG cascade will result in infertility.

Ovulation is a highly regulated flow of events is classified as inflammatory (Duffy et al., 2019) and prostaglandins play a vital role in inflammation. Inhibition of prostaglandin production would therefore interfere with inflammation and hence ovulation. Acetaminophen as discussed earlier inhibits COX production by competing with arachidonic acid (Botting 2000).

In this study, I analysed the effect of Acetaminophen on the oestrous cycle and hormone production in female mice.

4.2 MATERIALS AND METHODS

Daily vaginal washes were taken to determine cyclicity before the onset of the study. Only mice showing regular 4–5-day oestrous cycles were included in the study. Male mice were housed in separate cages within the laboratory. The mice were also examined daily for any signs of distress or toxicity. They were weighed daily and the weight was recorded.

4.2.1 EFFECT OF ACETAMINOPHEN ON OESTROUS CYCLE

Using ten normocyclic female mice divided into two groups (treatment and control) of five mice each, the effect of Acetaminophen on the oestrous cycle was assessed. The treatment group received 200 mg/kg of acetaminophen orally via gavage for twenty days (Hawk *et al.,* 2005). The mice in the control group were given 0.5ml of normal saline every day for 20 days in order to evaluate cyclic changes for 4-5 cycles.

Vaginal smears were collected daily through gentle lavage using phosphate-buffered saline, as described by Cora *et al*. (2015). The oestrous cycle was staged by examining the morphology and proportion of the leukocytes and the epithelial cells under an X40 objective light microscope (Cora et al., 2015). Early Proestrus was characterised by the presence of mainly nucleated epithelial cells and few if any cornified epithelial cells. Oestrus was identified by the predominance of cornified epithelial cells. Metestrus was associated with reducing the number of cornified epithelial cells and an increase in the number of nucleated epithelial cells and leukocytes. Dioestrus was mainly associated with leucocytes and nucleated epithelial cells. The stage of the cycle was recorded daily. The length of each stage and the entire cycle were calculated at the encd of the twenty (20) days. The experiment was repeated three times.

4.2.2 EFFECT OF ACETAMINOPHEN ON FEMALE REPRODUCTIVE HORMONES

The concentrations of female reproductive hormones were determined by this investigation using Laser Raman spectroscopy. Follicle-stimulating hormone (FSH), luteinizing hormone (LH), oestradiol, and progesterone were the hormones that were tested. A Raman spectrometer (STR, Airfix Corporation) confocal dispersive Raman spectrometer outfitted with a macro-Raman measuring attachment was used to conduct Raman experiments at ambient temperature (Easton, MD). A charge-coupled device detector that was thermoelectrically cooled and used to create Raman scattering was used. The quartz slide-mounted micro-Raman system enabled accurate measurements of solid materials. Samples were measured using various accumulations, scan periods, and slit sizes between 0.1 and 6 mm. The concentrations of female reproductive hormones were determined by this investigation using Laser Raman spectroscopy. Follicle-stimulating hormone (FSH), luteinizing hormone (LH), oestradiol, and progesterone were the hormones that were tested. A Raman spectrometer (STR, Airfix Corporation) confocal dispersive Raman spectrometer outfitted with a macro-Raman measuring attachment was used to conduct Raman experiments at ambient temperature

(Easton, MD). A charge-coupled device detector that was thermoelectrically cooled and used to create Raman scattering was used. The quartz slide-mounted micro-Raman system enabled accurate measurements of solid materials. Samples were measured using various accumulations, scan periods, and slit sizes between 0.1 and 6 mm.

For identification, calibration, and quantification using Raman spectroscopy, standard female reproductive hormones were combined with blood from male mice.

About 5µl of standard female reproductive hormone was measured and smeared on a silver-coated glass slide. The standard hormone concentrations used for calibration were five mIU/ml for FSH and LH, 20 pg/ml for oestradiol, and 0.2 ng/ml for progesterone.

The Raman spectrometry hormone assay required daily blood collection from the study mice's tail vein and application to a silver-coated slide. In accordance with Ondieki et al (2022) descriptions of feature selection in R software, Artificial Neural Networks (ANN) predictive models were created utilizing the detected spectral bands (2022). The hormone levels in the mice's blood from both groups of experiments were then determined using ANN models. The oestrous cycle stage was then recorded and examined in relation to the projected concentrations.

4.2.3 STATISTICAL ANALYSIS

Data were entered in Microsoft excel and then transferred to SPSS version 12.0 for windows (SPSS, Chicago, Illinois, USA) for analysis. Student t-test was used to examine the difference between groups and ANOVA was used to compare the means after three runs of the experiments. P-value ≤ 0.05 was considered statistically significant.

4.3 RESULTS

4.3.1 EFFECT OF ACETAMINOPHEN ON THE OESTROUS CYCLE

The mice in the study did not show any signs of distress for the twenty days of the experiment.

There was no difference in weight between the control and treatment groups for the entire duration of the study (Figure 3). There were fluctuations in the weight over the duration of the study but the trend was similar for both treatment and control groups.

There was no difference in the length and number of oestrous cycles observed over the 20 days in all the runs (Table 1 and Figure 4) between the control and treatment groups. However, there was a significant reduction in the frequency of the proestrus stage in the treatment group compared to the control group (Table 2 and Figure 5). There was no difference between the treatment and control group in all the other oestrous cycle stages.

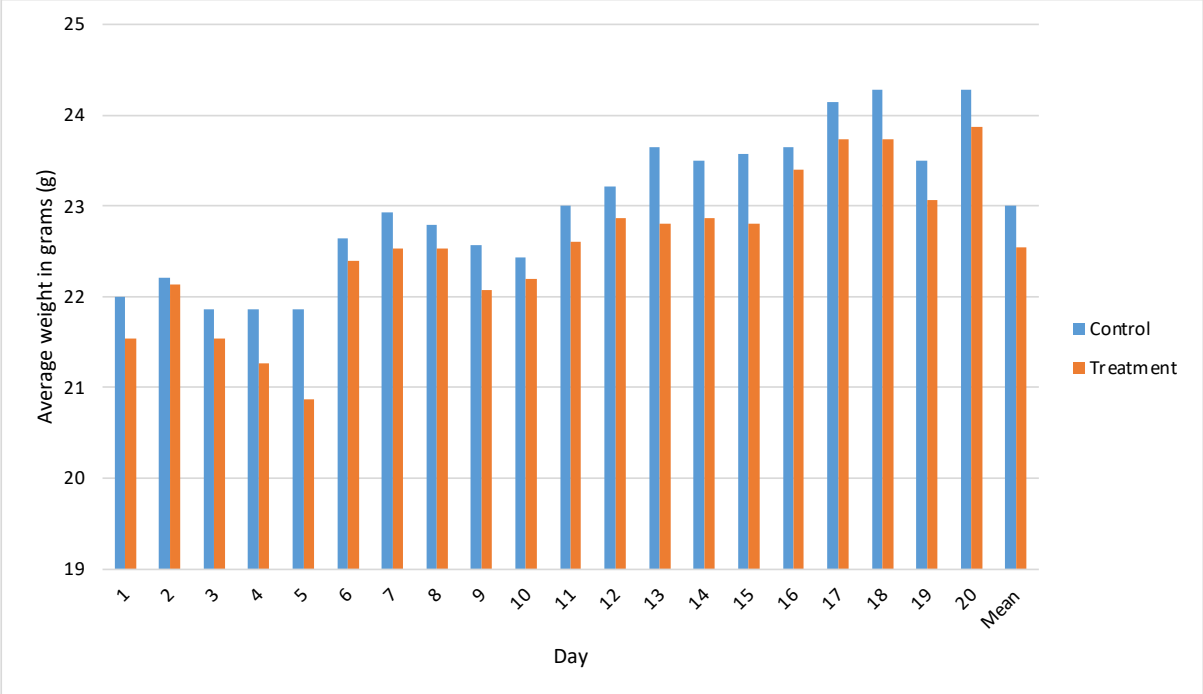

Figure 3: Mice's average daily weight in the treatment and control groups.

Figure 3 shows the average daily weight for mice in the control and treatment groups. The results show no difference in weight over the duration of the study.

Table 1: Average number of cycles observed over 20 days of treatment compared with the control

Parameter	Control	Treatment
Run 1	3.7 ± 0.274	3.0 ± 0.274
Run 2	3.2 ± 0.245	3.4 ± 0.245
Run 3	3.75 ± 0.274	3.2 ± 0.245
Average	3.54 ± 0.144	3.21 ± 0.155

Table 1 shows the number and length of oestrous cycles after 20 days of administration of acetaminophen at 200mg/Kg. The results are non-significant when compared to the control.

Mean number and length of estrous cycle expressed as Mean ± SEM (n=5/ group).

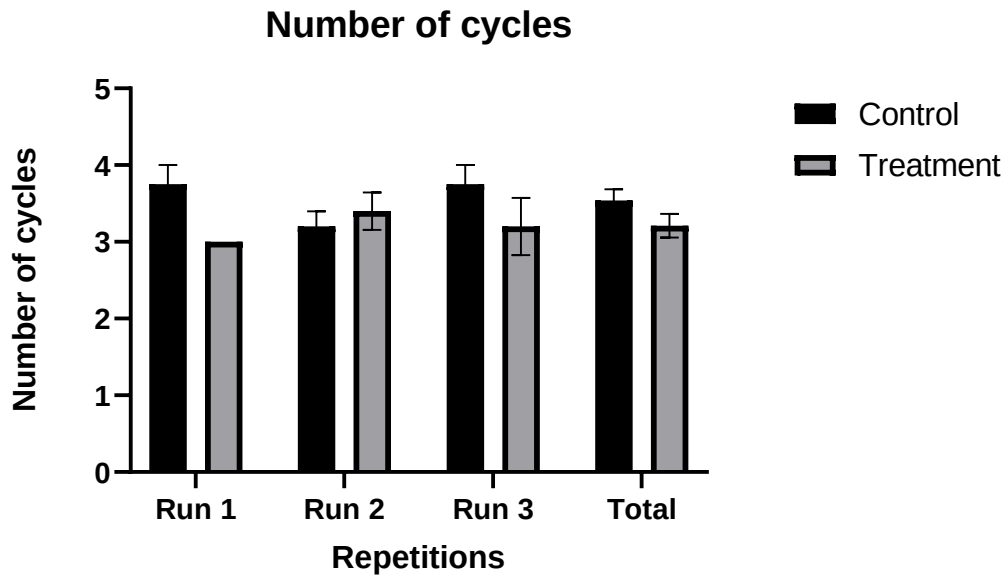

Figure 4: Average number of cycles after 20 days of treatment

Figure 4 shows the number of cycles recorded after 20 days of administration of acetaminophen at 200mg/Kg. The results are non significantt compared to the control. Mean number cycles expressed as Mean ± SEM (n=5/ group).

Table 2: Average length of oestrous cycle observed over 20 days

Parameter	Control	Treatment
Run 1	4.92 ± 0.32	5.75 ± 0.32
Run 2	5.86 ± 0.286	5.4 ± 0.286
Run 3	5.25 ± 0.32	5.47 ± 0.286
Average	5.38 ± 0.172	5.52 ± 0.187

Table 2 shows the length of oestrous cycles after 20 days of administration of acetaminophen at 200mg/Kg. The results are non-significant when compared to the control. Mean length of oestrous cycle expressed as Mean ± SEM (n=5/ group).

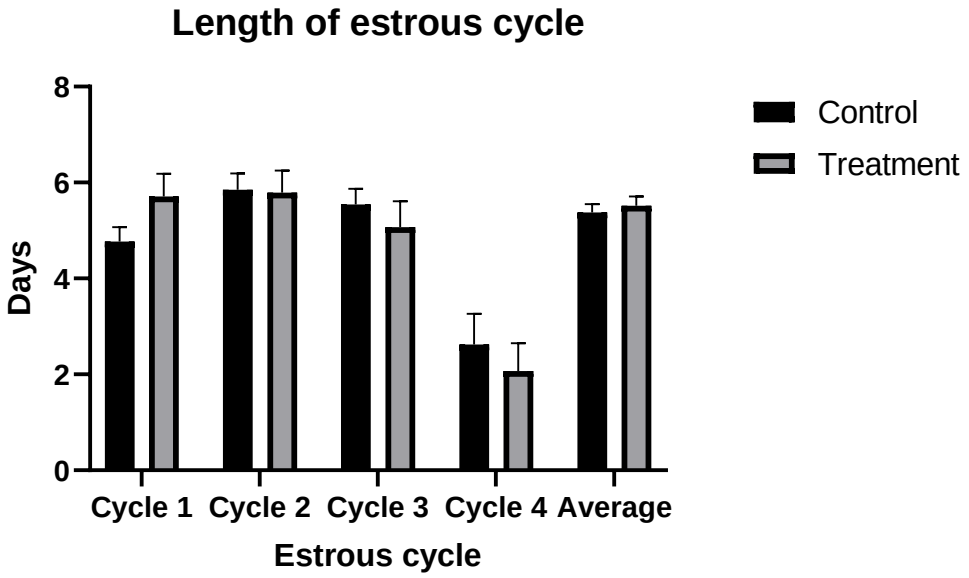

Figure 5: Average length of oestrous cycle.

Figure 5 shows the length of oestrous cycles after 20 days of administration of acetaminophen at 200mg/Kg. The results are non-significant when compared to the control. Mean length of estrous cycle expressed as Mean ± SEM (n=5/ group).

Table 3: Appearance frequency of oestrous cycle stages during the 20-day Acetaminophen administration. Results presented as X ± SEM

Stage of Estrous	Control	Treatment
Proestrus	4.77 ± 0.323	3.79 ± 0.281*
Oestrus	5.31 ± 0.365	6.21 ± 0.408
Metestrus	4.38 ± 0.266	4.36 ± 0.551
Dioestrus	5.23 ± 0.579	5.36 ± 0.599

Mean values are expressed as Mean ± SEM (n=5/ group). *** P< 0.001 **P<0.01 *P<0.05.

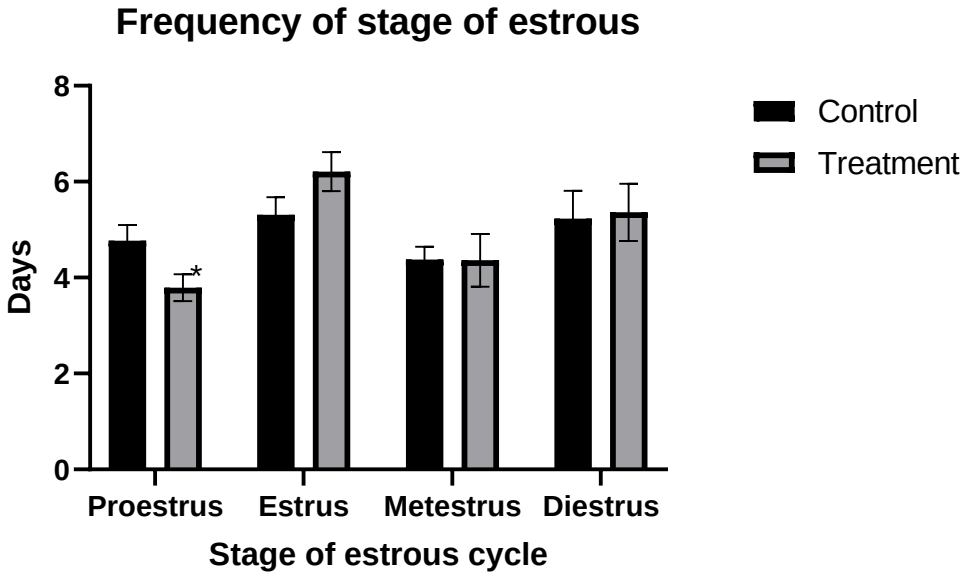

Figure 6: Frequency of stage of oestrous

Figure 6 shows the Frequency of appearance of the four stages of oestrous during the 20 days of administration of acetaminophen at 200mg/Kg. The results show a significant reduction in the frequency of proestrus compared to the control. Mean length of oestrous cycle expressed as Mean ± SEM (n=5/ group). *P<0.05.

4.3.2 EFFECT OF ACETAMINOPHEN ON FEMALE REPRODUCTIVE HORMONES

When analysed by group and stage of the oestrous cycle, no difference was observed in the oestradiol, FSH, and LH levels between the control and treatment groups (

Table 4). There was, however, a difference in the pattern of production for the three hormones in the treatment group with peak production during metestrus as opposed to oestrus as seen in the control group (Table 4). The levels and pattern of progesterone production did not differ between the two groups. However, there were significantly higher levels of progesterone during metestrus in both treatment groups $p \leq 0.034$.

Table 4: Raman spectral data table showing the average levels of female reproductive hormones in the treatment compared to the control group

Estrous cycle	FSH (mIU/mL)		LH (mIU/mL)		Oestradiol(pg/mL)		Progesterone(ng/mL)	
	Control (± 0.30)	Treated (± 0.46)	Control (± 2.77)	Treated (± 8.18)	Control (± 0.73)	Treated (± 0.72)	Control (± 0.87)	Treated (± 0.98)
Proestrus	3.033	3.413	114.59	101.59	8.939	9.591	12.08	9.252
Oestrus	3.666	2.890	125.39	102.73	10.21	9.029	13.39	10.41
Metestrus	3.500	4.900	119.34	136.67	6.728	12.10	15.27	13.79
Dioestrus	2.341	3.085	113.13	110.31	9.130	9.166	11.29	11.80

There was no significant difference in the amount of follicle-stimulating hormone (FSH) produced in the control and treatment groups (Table 5). However, there was a disruption in the pattern of production with peak FSH production seen in metestrus compared to oestrus in the control group (Figure 7).

Table 5: Raman spectral data table showing the average levels of Follicle-stimulating hormone in mIU/m compared to the control

Oestrous stage	Control	Treatment
Proestrus	3.69 ± 0.89	3.44 ± 0.93
Oestrus	3.39 ± 0.66	2.74 ± 0.7
Metestrus	3.12 ± 0.86	5.22 ± 0.78
Dioestrus	2.24 ± 0.81	3.17 ± 0.83
Overall	3.11 ± 0.41	3.64 ± 0.41

Table 5 shows the levels of Follicle stimulating hormone in the four phases of estrous. There was non-significant difference in the production levels between the control and treatment groups. Mean values are expressed as Mean ± SEM (n=5/ group).

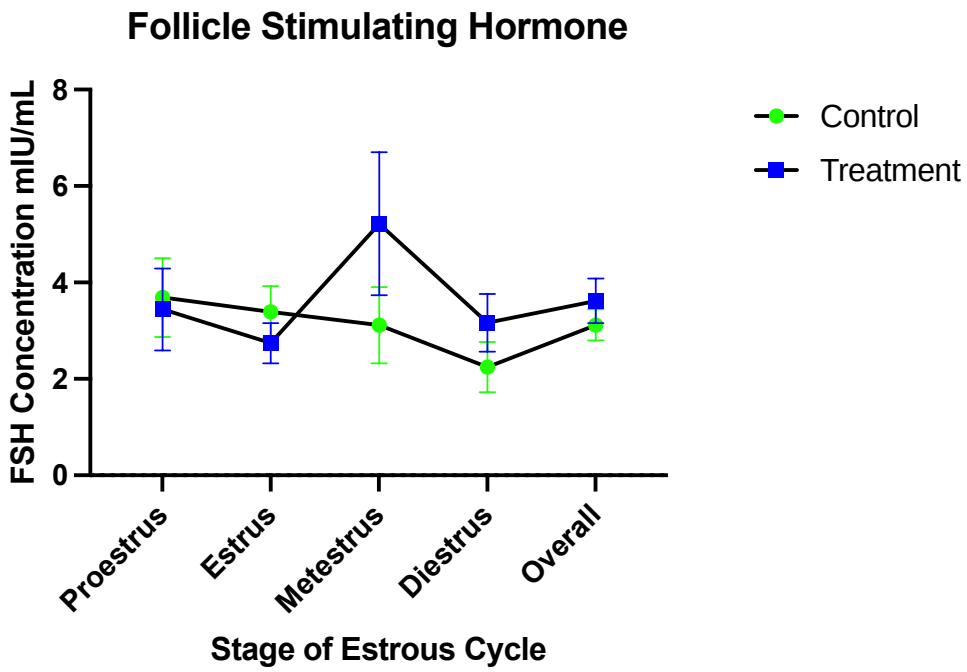

Figure 7: Production pattern of Follicle Stimulating hormone by stage of the oestrous cycle

Figure 7 shows the production pattern of Follicle stimulating hormone. The figure shows there was a delay in the FSH surge in the treatment group with peak production in metestrus compared to oestrus in the control group. There was however no significant difference in the overall FSH production levels.

Table 6: Raman spectral data table showing the average levels of Luteinizing hormone in mIU/mL compared to the control

Oestrous stage	Control	Treatment
Proestrus	9.17 ± 1.341	9.17 ± 1.60
Oestrus	10.24 ± 1.0	10.43 ± 1.16
Metestrus	7.57 ± 1.34	12.35 ± 1.41
Dioestrus	9.19 ± 1.23	8.87 ± 1.31
Overall	9.04 ± 0.617	10.20 ± 0.69

Table 6 shows the levels of Luteinizing hormone in the four phases of oestrous. There was non-significant difference in LH production levels between the control and treatment groups. Mean values are expressed as Mean ± SEM (n=5/ group).

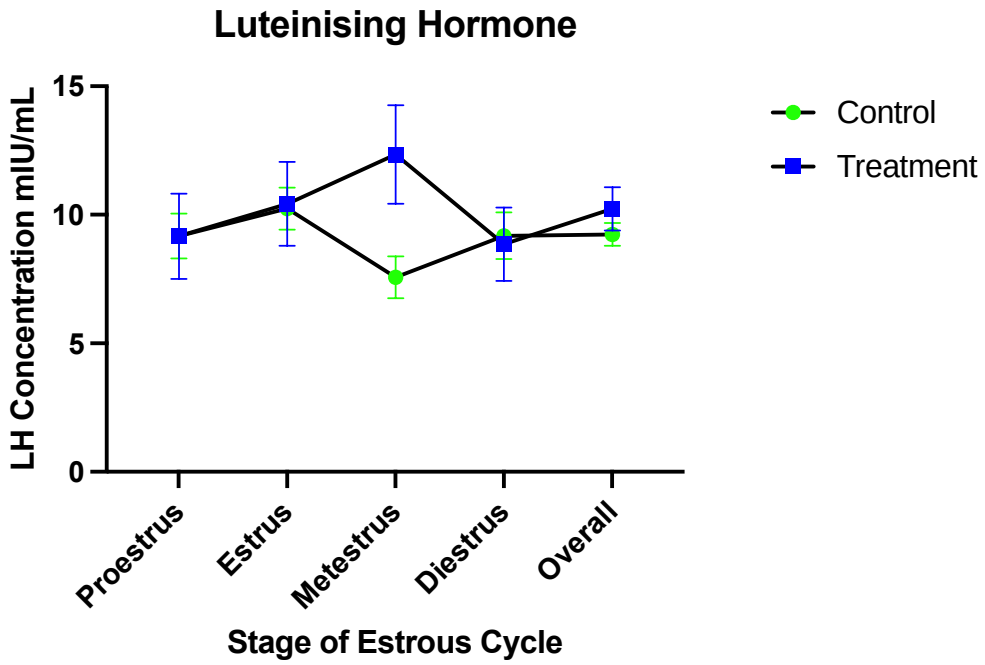

Figure 8: Production pattern of Luteinizing hormone by stage of the oestrous cycle

Figure 8 shows the production pattern of the Luteinizing hormone. The figure shows there was a delay in the LH surge in the treatment group with peak production in metestrus compared to oestrus in the control group. There was no difference in the overall production levels between the control and the treatment group.

There was non-significant difference in the levels of oestrogen produced between the treatment

and control groups when the levels were analysed by stage of the oestrous cycle and group (

Table *7*). However, peak oestrogen production was seen during metestrus in the treatment group as opposed to it being observed during oestrus in the control group (Figure 9).

Table 7: Raman spectral data table showing the average levels of Oestrogen in pg/mL compared to the control

Estrous stage	Control	Treatment
Proestrus	119.45 ± 9.5	101.65 ± 10.01
Oestrus	123.72 ± 7.08	102.7 ± 7.51
Metestrus	114.63 ± 9.5	131.70 ± 8.33
Dioestrus	114.96 ± 8.67	110.7 ± 8.86
Overall	118.19 ± 4.37	111.69 ± 4.36

Table 7 shows the levels of Oestrogen hormone in the four phases of estrous. There was non-significant difference in oestrogen production levels between the control and treatment groups. Mean values are expressed as Mean ± SEM (n=5/ group).

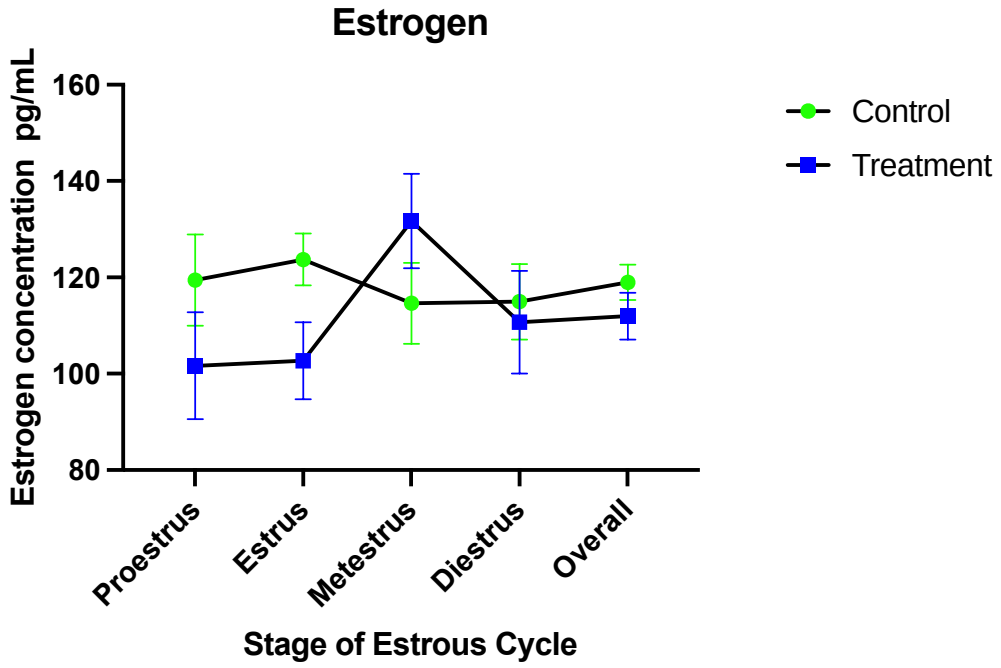

Figure 9: Production pattern of Oestrogen by stage of the oestrous cycle

Figure 9 shows the production pattern of oestrogen hormone. The figure shows there was a delay in the oestrogen surge in the treatment group with peak production in metestrus compared to oestrus in the control group. There was no difference in the overall production levels.

There was significant reduction in the overall levels of progesterone in the treatment group compared to the control (Table 8). There was however, no difference in the average level of progesterone between the two groups when analysed according to stage of the cycle. The production pattern of progesterone was similar in the control and treatment groups (Figure 10).

Table 8: Raman spectral data table showing the average levels of Progesterone in ng/mL compared to the control

Estrous Stage	Control	Treatment
Proestrus	12.69 ± 1.07	9.45 ± 1.23
Oestrus	13.13 ± 0.8	10.32 ± 0.85
Metestrus	13.15 ± 1.07	12.86 ± 0.94
Dioestrus	11.66 ± 0.98	12.0 ± 1.0
Overall	12.66 ± 0.49	11.16 ± 0.49*

Table 8 shows the levels of progesterone hormone in the four phases of oestrous. There was a significant difference in the overall production levels between the control and treatment groups. Mean values are expressed as Mean ± SEM (n=5/ group). *P<0.05.

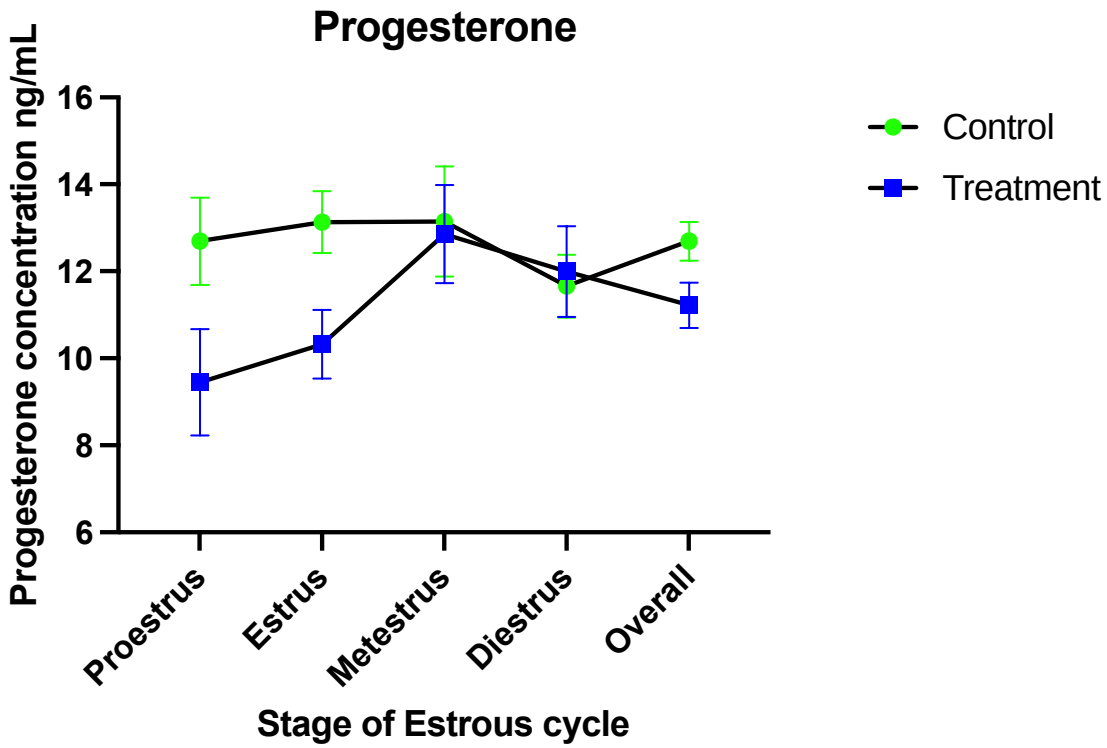

Figure 10: Production pattern of progesterone by stage of the oestrous cycle

Figure 10 shows progesterone production pattern. The production pattern was similar for both groups however, there was a significant lower level of progesterone in the treatment compared to the control group.

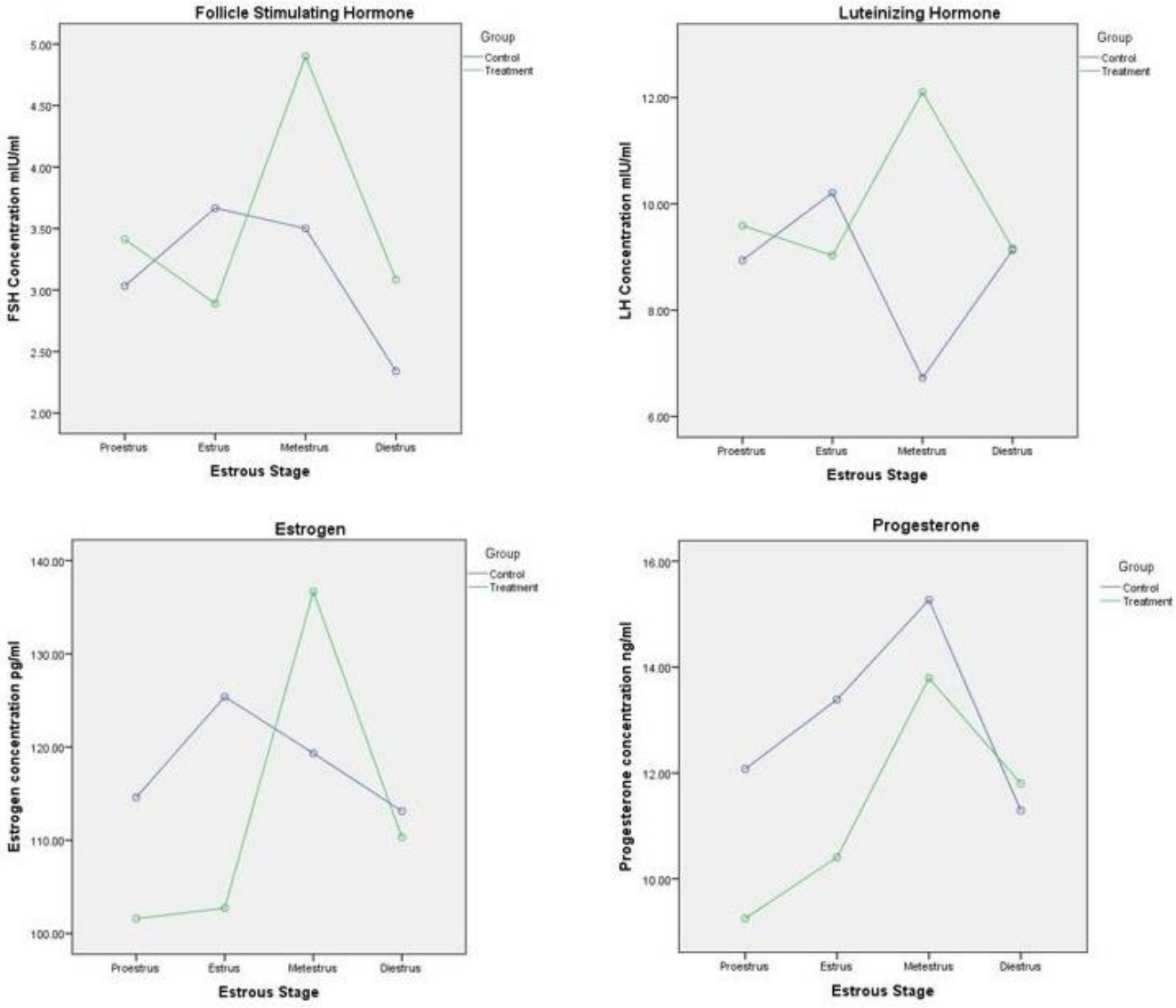

Figure 11: Production pattern of female reproductive hormones by stage of the oestrous cycle

Figure 11 shows the production pattern of all four female reproductive hormones. The figure shows there was a delay in the FSH, LH and oestrogen surge in the treatment group with peak production in metestrus compared to oestrus in the control group. The production pattern was similar for progesterone in both groups.

Two mice were lost in the course of the study due to follow-up. This happened because one mouse got pregnant before the intended day of mating and had to be excluded from the study. The second mouse was non-cyclic and had a prolonged dioestrus period and had to be removed from the study.

4.4 DISCUSSION

The mice in this study had consistent oestrous cycles over the course of the whole 20-day investigation. The average length of the oestrous cycle in mice is four to five days (Cora *et al.,* 2015). The length and quantity of oestrous cycles did not differ between the control and treatment groups. However, the length of the oestrous cycle was greater than the average; this may be linked to housing. In this study, it was discovered that mice housed alone have more regular and consistent 4/5-day cycles than mice kept in groups (Cora *et al.,* 2015). According to this study, the oestrous cycle consists of four phases: proestrus, oestrus, metestrus, and dioestrus. This study's use of wet mounts for direct microscopy to measure oestrous cycle stages is consistent with previous research (Caligioni, 2009; Cora *et al.*, 2015).

Significantly less proestrus occurred in the treatment group compared to the control group. Each stage lasts approximately 6 to 72 hours, depending on the stage and mouse. In mice, proestrus lasts approximately 12 to 24 hours. There is a possibility that brief stages, such as proestrus, may be'missed' if sampling occurs too early or too late in the day (Cora *et al.,* 2015). This appeared improbable, however, given that sampling occurred simultaneously for both groups, however the drop was found only in the treatment group. The most plausible explanation is that follicles in the therapy group had premature luteinisation, leading the proestrus phase to conclude prematurely. When antral follicles fail to ovulation, luteinized unruptured follicles (LUFs) frequently occur (Bashir *et al.,* 2016). Several prostaglandin inhibitors have been demonstrated to cause luteinisation of follicles in both animals and women (Bashir *et al.,* 2018), and as acetaminophen reduces prostaglandin synthesis, this is likely to have occurred. Specifically, a prolonged LH surge, as observed in this study (Figure 8), is one of the reasons of LUF (Bashir *et al.,* 2016). Prostaglandins create proteolytic enzymes that promote follicular rupture during regular cycles (Kim *et al.,* 2014). Therefore, acetaminophen may have produced prolonged follicles and ovulatory failure by blocking the COX enzyme.

Acetaminophen had no effect on the levels of FSH, LH, and Oestradiol; other NSAIDs investigations found the same thing (Al-Atraki et al., 2012; Martini et al., 2008). Hypothalamic gonadotropin-releasing hormone causes the anterior pituitary to release FSH and LH (Christensen *et al.,* 2012). Granulosa cells of growing follicles generate estrogen when stimulated by FSH. During proestrus, oestradiol levels typically increase gradually, peaking right before ovulation. During metestrus, oestradiol levels decrease before rising again during dioestrus (Caligioni, 2009; Ndeke et al., 2022). This rapid increase in plasma oestradiol concentration during proestrus promotes an increase in GnRH production via a positive feedback process (Miller and Takahashi, 2014). In turn, GnRH stimulates anterior pituitary gland LH production, which in turn stimulates ovulation (Miller and Takahashi, 2014). In this study, the FSH, LH, and estrogen levels were abnormally high during metestrus in the therapy group. The observed changes suggest that this cascade was delayed, with peak hormone levels occurring during metestrus as opposed to oestrus. This delay may have resulted in a delayed ovulation. One of the detrimental consequences of acetaminophen in mice is delayed ovulation, which reduces the number of implantation sites (Ndeke et al., 2021).

Suppression of LH production in the late luteal phase has been shown to terminate early pregnancies by reducing ovarian production of progesterone. The findings of this study agree with this because when administered prior to mating, acetaminophen significantly reduced the number of implantation sites in the treatment group (Ndeke et al., 2021). While the levels of LH were not directly suppressed, by delaying the LH peak, acetaminophen is likely to have delayed ovulation and resulted in reduced progesterone production, as was observed in this study. This interference in LH production has been documented in the use of marijuana, indicating that there is a likelihood that acetaminophen interfered with hormone production by activating the endocannabinoid system, similar to Δ-9-THC (BRENTS, 2016).

There was significantly lower level of progesterone in the treatment group compared to the control. Progesterone is primarily produced by the corpus luteum before the function being taken up by the placenta during pregnancy. There is a likelihood of corpus luteum insufficiency in this study. Corpus luteum insufficiency is associated with the abnormal formation of ovarian follicles. These abnormalities may be so pronounced that no secondary or tertiary follicles are produced, resulting in anovulation (Murray, 2020). Corpus luteum insufficiency also leads to a relative deficiency of progesterone as was seen in this study.

Raman spectroscopy has drawn attention in biological sample analysis (Liu et al., 2018; Vedad et al., 2018); however, the raw Raman spectral data obtained is vulnerable to background influences and fluorescence that a pre-processing stage should extract (Kuhar et al., 2018; Ondieki et al., 2022). These results have shown that Raman spectroscopy and chemometrics can measure hormone levels in the blood as was used in other studies (Duraipandian et al., 2013; Y. Liu et al., 2018; Ondieki et al., 2022; Vedad et al., 2018).

4.5 CONCLUSION

The effect of acetaminophen on reproductive hormones indicates its possible contraceptive properties of acetaminophen by delaying production of LH, FSH and oestrogen. The exact mechanism and impact of this delay should be explored further. The effect might either be in the hypothalamus, pituitary or ovaries. In this chapter, the study has successfully evaluated the effect of acetaminophen on female reproductive hormones. A manuscript has been submitted for peer review in the *international journal of veterinary sciences.*

Ndeke AN, Mutembei HM, Kaingu CK, Muthee JK, Birech Z and Ondieki AM, 2022. Effect of acetaminophen on the estrous cycle and reproductive hormones of female mice. International Journal of Veterinary Science 11(3): 392-395. https://doi.org/10.47278/journal.ijvs/2021.118

CHAPTER 5

5.0 EFFECT OF ACETAMINOPHEN ON IMPLANTATION IN FEMALE MICE

5.1 INTRODUCTION

Implantation is the process where the blastocyst makes intimate physiological and physical contact with the endometrium (Dey et al., 2004). The quality of the blastocyst, the presence of a receptive endometrium, and the synchronisation of the embryo's developmental stages all contribute to successful implantation. Successful implantation is dependent on the availability of a competent embryo and a receptive uterus (Wang and Dey, 2006).

Implantation is a complicated process involving the coordinated actions of autocrine, paracrine, and endocrine systems and is influenced by various factors, some of which are still unknown (Singh et al., 2011). As a result, implantation failure might be caused by poor blastocyst quality or endometrial receptivity. Animal models have revealed important information about the molecular pathways during embryo implantation (Dey et al., 2004; H. Wang et al., 2003). The growth of an embryo to the blastocyst stage and the formation of an endometrium that is receptive to the embryo are both required for successful implantation (Kubota et al., 2016). In rodents and humans, the endometrium is known to become responsive for only a brief duration known as the ‘implantation window.’ The embryo cannot correctly establish contact with a refractive endometrium once this period of receptivity has passed.

After fertilisation, locally acting hormones and growth factors mediate the initial communication between the free-floating blastocyst and the receptive endometrium (Lopata et al., 2002). The Endocannabinoid System has been shown to regulate implantation through the synchronous crosstalk between the endometrium and the embryo to guarantee timely and successful implantation (Bambang et al., 2010). Endocannabinoids (ECS) ligands are known to work through cannabinoid receptor type 1 (CB1) and 2 (CB2) and related enzymes, which have been demonstrated to be present in females (Cui, et al., 2017; Fride, 2002).

Acetaminophen undergoes metabolic deacetylation in the liver to yield p-aminophenol (Högestätt et al., 2005; Mazaleuskaya et al., 2015), which is further conjugated with arachidonic acid by action of fatty acid amide hydrolase (FAAH), resulting in the formation of N-arachidonoyl-phenolamine (AM404) in the CNS (Saliba et al., 2017; Smith, 2009; Zygmunt et al., 2000). It is the AM404 which selectively binds to CB1 receptors that activates the endocannabinoid system, this binding to CB1 receptors inhibits cellular re-uptake of anandamide which allows a build-up of anandamide levels within the extracellular space (Fegley et al., 2004; Högestätt et al., 2005; Ruggieri et al., 2008). By acting on the endocannabinoid system it was postulated that acetaminophen may affect some female reproductive processes, probably at the levels of ovulation and implantation (Cui, et al., 2017; Di Marzo and Deutsch, 1998; Maccarrone, De Felici, et al., 2000; Paria et al., 2001; Schmid et al., 1997; Yang et al., 1996).

This experiment was carried out in mice to shed further light on the potential roles of acetaminophen on female reproduction.

5.2 MATERIALS AND METHODS

5.2.1 EVALUATING THE EFFECT OF ACETAMINOPHEN ON IMPLANTATION SITES

Two experiments were used to study the effect of acetaminophen on implantation in mice.

5.2.1.1 Pre-mating acetaminophen treatment regime

Mice received either Acetaminophen (treatment group) or normal saline (control group) daily via oral gavage for seven days before mating and continued receiving treatment until the 7th day of pregnancy. The treatments were administered in the morning between 7:00 and 9:00 am. Mice in the control group received normal saline while those in the treatment group received 200mg/kg acetaminophen by oral gavage for seven days. On the seventh day of treatment, males of proven fertility were introduced into the cages at a ratio of 1:2 i.e. one male per two

females. The males used in this study had been used previously for breeding hence proof of fertility prior to use. The mice were then monitored every morning for evidence of mating. The day a vaginal plug was seen, was considered the first day of pregnancy. The mice were sacrificed on the seventh day of pregnancy, and the uteri were harvested to count the number of implantation sites and thereafter preserved in 10% formalin for future analysis.

5.2.1.2 Post mating acetaminophen treatment regime

Female mice were first housed with males of proven fertility at a ratio of 1:2. The males used were of proven fertility as stated in 5.2.2.1 above. The mice were monitored every morning for evidence of mating (Vaginal plug). The day a vaginal plug was seen, was considered day one of pregnancy. Once the plug was seen treatment was started on the same day. Treatment was given daily via oral gavage between 0700-0900 hours from the 1st to 7th day of pregnancy. Mice in the control group received normal saline while those in the treatment group received 200mg/kg acetaminophen by oral gavage. On the seventh day of pregnancy, the mice were sacrificed and the uteri harvested to count the number of implantation sites and preserved for future analysis. Both experiments were repeated three times (n=3).

5.2.2 EFFECT OF ACETAMINOPHEN ON NUMBER OF OFFSPRING

Female mice were housed with males of proven fertility at a ratio of 1:2. Daily vaginal flushing was done using physiological saline to determine the presence of spermatozoa. The day a vaginal plug was seen was considered day one of pregnancy. Treatment was given via oral gavage daily between 0700-0900 hours from the 1st to 7th day of pregnancy. The mice were then allowed to carry the pregnancies to term, and the number of pups born was counted. The experiment was repeated twice.

5.2.3 REVERSIBILITY OF THE EFFECT OF ACETAMINOPHEN ON IMPLANTATION

The mice used in 5.2.3 were used for this experiment. The pups born were sacrificed immediately after delivery. The mice were then given two weeks to resume cyclicity. They were then housed with males of proven fertility and allowed to mate. The day a vaginal plug was seen was considered the first day of pregnancy. The mice did not receive any intervention and were sacrificed on the seventh day of pregnancy to count the implantation sites.

5.2.4 DATA HANDLING

Data from all the mice in each experimental group (implantation sites, litter size) was computed to obtain a group mean (mean±SD) and the mean of all means of the repeated experiments was calculated to obtain the mean of the mean (mean±SEM). Statistical difference between the mean of treatment and control group was tested using a student's t-test at a 95% confidence interval. Analysis of variance (ANOVA) was used to compare means in the three runs of the experiments.

5.3 RESULTS

5.3.1 EFFECT OF ACETAMINOPHEN ON IMPLANTATION

All mice in both experiments were mated successfully, and a vaginal plug was observed. There was no difference in the rate of pregnancy in the treatment and control group when acetaminophen and normal saline were administered pre-coital. Both groups had a pregnancy rate of 70%. The mice were considered pregnant by the presence of at least one visible implantation site. The total number of implantation sites were also manually counted and recorded after harvesting the uterus.

There was a significant reduction of implantation sites between the treatment groups when acetaminophen was administered before mating ($P \leq 0.05$) when compared with the respective control groups (Table 9).

Table 9: Counted Implantation Sites of the pre-coital administration of acetaminophen

Parameter	Control	Treatment
Run 1	7.2 ± 4.147	5.0 ± 4.64
Run 2	7.4 ± 7.34	4.2 ± 5.848
Run 3	8.8 ± 3.271	1.8 ± 4.025*
Mean of Means (m± SEM)	7.8± 1.306	3.67± 1.306*

Table 9Mean implantation values are expressed as Mean ± SEM (n=5/ group). *** P< 0.001 **P<0.01 *P<0.05.

When administered after mating, acetaminophen significantly reduced the pregnancy rate to 40% ($p < 0.01$) in the treatment group compared to the control that had a 100% pregnancy rate. Acetaminophen also significantly reduced the number of implantation sites when administered for seven days post-mating (Table 10),

Table 10: Number of recorded implantation sites following administration of acetaminophen/normal saline for seven days post-mating

Parameter	Control	Treatment
Run 1	10 ± 3.6	1.6 ± 3.57**
Run 2	9.6 ± 1.5	1.8 ± 4.02**
Run 3	10.6 ± 3.3	3 ± 4.12*
Mean of Means	10.06 ±0.898	2.13± 0.898***

Table 10 shows the anti-implantation activity of post-mating administration of acetaminophen at 200 mg/Kg. The results show a significant reduction in the number of implantation sites in all runs compared to the control. Mean implantation values are expressed as Mean ± SEM (n=5/ group). *** P< 0.001 **P<0.01 *P<0.05.

5.3.2 EFFECT OF ACETAMINOPHEN ON NUMBER OF OFFSPRING

There was a significant reduction in the number of offspring born after administration of acetaminophen for seven days post-mating (Table 11 and figure 12).

Table 11: Number of off-springs born following administration of acetaminophen/normal saline for seven days post-mating.

Parameter	Control	Treatment
Run 1	9.4 ± 1.140	2.2 ± 3.493*
Run 2	9.4 ± 0.894	4 ± 3.082*
Mean of Means	9.4 ± 0.306	3.1 ± 1.027*

Table 11 shows the mean number of off springs born after post-mating administration of acetaminophen at 200 mg/Kg. The results were significant in each run compared to the control. Mean off spring values are expressed as Mean ± SEM (n=5 group). *P<0.05.

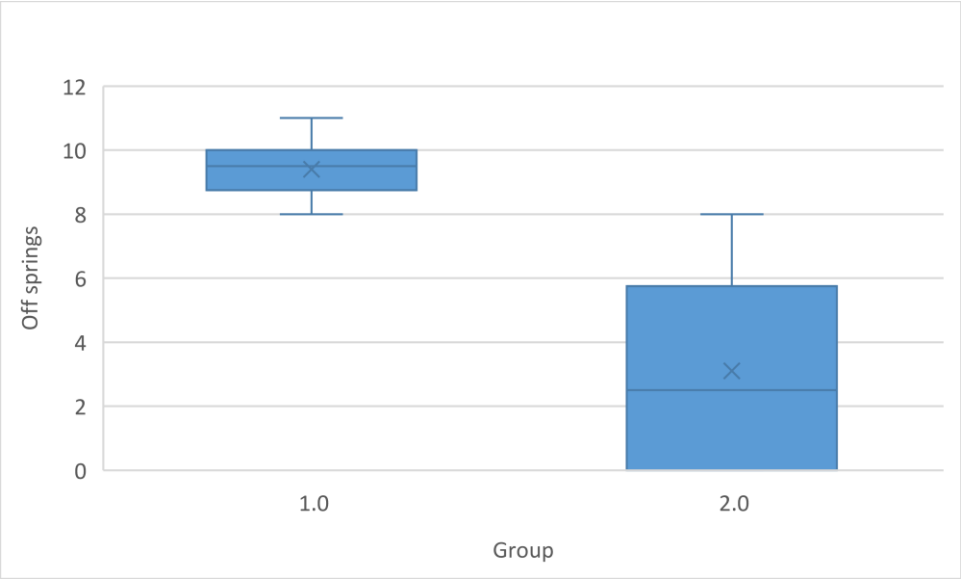

Figure 12: Effect of acetaminophen on the number of offspring born after seven-day treatment and withdrawal of acetaminophen/normal saline.

Mean off-spring values are expressed as Mean ± SEM (n=5/ group). *** P< 0.001 **P<0.01 *P<0.05.

5.3.3 REVERSIBILITY OF THE EFFECT OF ACETAMINOPHEN ON IMPLANTATION

After the withdrawal of acetaminophen and housing with males of proven fertility, all females in the control and treatment groups mated successfully. There was a non-significant difference in the number of implantation sites between the treatment and control groups (

Table 12).

Table 12: Average number of implantation sites after treatment and withdrawal of acetaminophen/Normal saline

Parameter	Control	Treatment
Run 1	5.8 ± 1.817	8.6 ± 1.817
Run 2	6.4 ± 1.187	9.2 ± 1.817
Mean of Means	7.2 ± 1.285	7.8 ± 1.285

5.4 DISCUSSION

A significant reduction in the number of implantation sites ($p<0.0005$) was observed in the treatment groups compared to the control groups. This reduction suggests a potential role of acetaminophen in regulating ovulation and implantation in female mice.

By administering acetaminophen pre-coitus, there is a likelihood of interference with the oestrous cycle, as discussed in chapter 5. The interference might have impaired ovulation resulting in low implantation sites in the treatment group. Prostaglandins play a vital role in the ovulation process, which is considered an inflammatory event. Inhibition of prostaglandin synthesis by acetaminophen may have resulted in impaired ovulation. Other NSAIDs linked with impaired ovulation include indomethacin, ibuprofen and celecoxib (Albert et al., 2013; Edelman et al., 2013; Ekanem et al., 2008; Kanayama et al., 1996; Kohl Schwartz et al., 2020; Sookvanichsilp and Pulbutr, 2002). This finding and the reduced frequency of proestrus in the treatment group seem to corroborate the theory that acetaminophen impaired ovulation in the treatment group (Ndeke *et al.,* 2021).

When administered post-mating, acetaminophen resulted in significantly lower implantation sites. There are three likely events that acetaminophen might have interfered with (I) embryo transport, (ii) decidualisation and (iii) implantation. Successful implantation depends on a competent embryo within the uterus and a uterus that is ready for implantation. Both prostaglandins and endocannabinoids play vital roles in embryo transport. Successful embryo transport within the oviduct depends on muscular contractility and ciliary movement (Anamthathmakula and Winuthayanon, 2021; Salleh, 2014; Spilman and Harper, 1975). A delicate balance of smooth muscle contraction and relaxation is needed to achieve this (Salleh, 2014). Both PGE and PGF2 play a role in these events of relaxation and contractility, respectively.

Similarly, aberrant anandamide signalling, occasioned by the administration of acetaminophen, might have affected embryo transport. In humans, users of marijuana have been demonstrated to have a higher incidence of ectopic pregnancies than non-users (Schuel, 2006). Therefore, the inhibition of prostaglandin production by acetaminophen may have resulted in impaired embryo transport translating to the low implantation sites observed in the treatment group.

Implantation is a highly regulated rate-limiting step in the establishment of pregnancy. It is likely that administration of acetaminophen during the implantation window caused an increase in anandamide levels, which compromised implantation. It has been demonstrated that the levels of anandamide at the implantation sites need to be lowered for successful implantation (Cui, et al., 2017). Anandamide has both direct and indirect effects on the developing embryo. High levels are detrimental to embryo survival. Anandamide causes an increase in tumour necrosis factor, an embryotoxic cytokine, which could likely affect embryo survival. In addition, anandamide lowers the levels of IL-4 and IL-6, which are embryo trophic in nature (Greco et al., 2020). A reduction in embryotropic cytokines and an increase in embryotoxic cytokines results in poor embryo survival.

A conducive environment is a prerequisite for successful implantation. Anandamide and prostaglandins play a role in the decidualisation of the uterus for implantation. Non-steroidal anti-inflammatory drugs such as indomethacin delay or inhibit the localised increase in endometrial vascular permeability and implantation in various species (Kennedy, 1980; Kennedy et al., 2007; Scherle et al., 2000). Hence, the low number of implantation sites in this study may be attributed to impaired decidualisation since acetaminophen, like indomethacin, inhibits prostaglandin production. In addition, the endocannabinoid system also plays a critical role in decidualisation. Elevated levels of anandamide at the time of implantation can result from either suppression of FAAH or the use of pharmacological compounds such as acetaminophen. These high levels have been shown to cause embryo retention within the

oviduct, inhibit embryo development and expression of cell lineage genes required for differentiation, compromise implantation, and cause an overall reduction in fertility (Fonseca et al., 2015). There is an 'estrogen surge' in mice and rats on the morning of day 4 of pregnancy; ovariectomy before this surge results in delayed implantation.

Evidence from this study seems to agree with others that acetaminophen impairs female reproduction through its action on the endocannabinoid system. Hence acetaminophen regulates both ovulation and implantation (Bisogno et al., 2000; Cui, Yang, et al., 2017; Devane et al., 1992; Fride, 2002; Mechoulam et al., 1995; Petrocellis et al., 2004; Porter et al., 2002; Sugiura et al., 1995). These results seem to agree with the evidence in in-vitro studies (Bambang et al., 2010). As seen in this study, the main anti-fertility effect of acetaminophen the anti-implantation could be its anti-implantation property. The post-mating treatment regime had the highest effect on fertility.

There was no difference in the number of implantation sites upon withdrawal of acetaminophen. This similarity shows that the withdrawal of treatment can reverse the effects of acetaminophen on reproduction. This temporary effect of acetaminophen on mice is similar to those reported for other non-steroidal anti-inflammatory drugs (Marrs et al., 2004; Stone S et al., 2002). As postulated to have occurred in this study, high levels of anandamide have also been attributed to reversible infertility in sheep (Turco et al., 2008). However, anandamide causes arrest of cell proliferation within the embryo, which, upon withdrawal, there is the resumption of growth (Turco *et al.,* 2008). It is unlikely that acetaminophen resulted in the arrest of cell proliferation within the embryo. If this were the case, it would not have affected the number of offspring born after administration for seven days post-mating. Upon withdrawal of treatment on the seventh day of pregnancy, the embryos should have started proliferating again, resulting in a similar number of offspring between the two groups. An ideal contraceptive should be reversible and not interfere with future reproduction. In this study, the withdrawal of

acetaminophen allowed the mice to return to normal cyclicity, indicating that it can be used as a contraceptive.

5.5 CONCLUSION

The effect of acetaminophen on implantation points toward its potential use as a contraceptive. From the results, acetaminophen had an anti-implantation effect when administered pre-and post-mating. These effects might either be in the ovaries and/or uterus or probably on reproductive hormones. In this chapter, the study has successfully evaluated the effect of acetaminophen on implantation and the number of offspring born. A manuscript has been published in the *International Journal of Veterinary Sciences.*

Ndeke AN, Mutembei HM, Kaingu CK and Muthee JK, (2021). Effect of acetaminophen on ovulation and implantation in female mice. International Journal of Veterinary Science 10(2): 141-143. https://doi.org/10.47278/journal.ijvs/2020.029

CHAPTER 6

6.0 THE EFFECT OF ACETAMINOPHEN ON THE HISTOMORPHOLOGY OF THE OVARIES AND ENDOMETRIUM

6.1 INTRODUCTION

Female fertility is dependent on the ability to produce mature oocytes that can be successfully ovulated, fertilised and develop into an implantation competent embryo (Kim and Kim, 2017). The uterus and ovary change with every oestrous cycle in preparation for pregnancy. These changes are mitigated by hormones from the hypothalamo-pituitary-gonadal axis. Follicle-stimulating hormone and Luteinizing hormone play a vital role in follicular development and ovulation. Any disturbance in the production of these hormones will directly affect folliculogenesis and eventually cause infertility. As discussed in section 4.4 above, acetaminophen lowered progesterone production and delayed the production of FSH, LH and Oestrogen might interfere with follicular development.

This study's results indicate that acetaminophen negatively affects female reproduction by inhibiting/delaying ovulation, further correlating the results in chapter 4 above. Further research is recommended to establish the exact mechanism of action.

6.2 MATERIALS AND METHODS

This study examined the effect of acetaminophen on the histomorphology of the ovary and uterus. The study utilised female Swiss white mice aged between 6 and 8 weeks. Mice in the treatment group received 200mg/kg of Acetaminophen by oral gavage while those in the control group received 0.5ml of normal saline for 20 days. At the end of the treatment period the mice were humanely euthanised and the uterus and ovaries harvested. The samples were stored in 10% formalin awaiting histological analysis.

This study utilised ovarian and uterine tissues harvested from mice in the two studies described above: effect of acetaminophen on the oestrous cycle: mice in this study were sacrificed during proestrus. The uteri and ovaries were extracted and preserved in 10% formalin for histological analysis. Effect of acetaminophen on ovulation and implantation: The ovaries and uteri were preserved in 10% formalin after counting the number of implantation sites on the seventh day of pregnancy.

Ovaries and uterine horns from sacrificed mice were harvested, fixed in 10% formalin, and processed for histology and morphometry as per the protocol described by (Slaoui and Fiette, 2011). A comparison was made between the treatment and control groups. There was blinding of the laboratory technician; the investigator organised the samples into 'Group A and B and Animals 1,2,3 for the treatment and control groups, respectively, so that the person doing the analysis knew which animals were grouped together but not what treatment the animals received. Some of the parameters evaluated include the presence or absence of degenerative changes in the ovaries, such as disruption of the zona pellucida, condensation of granulosa cells, and the number of corpora lutea. Uterine changes evaluated include thickening of the endometrial lining, presence or absence of villi, pyknotic cells, and vacuolation within the stroma.

6.3 RESULTS

6.3.1 EFFECT OF ACETAMINOPHEN ON HISTOMORPHOLOGY OF THE OVARY AND ENDOMETRIUM

There was no difference in the number of primary and antral follicles observed in the control and treatment groups. There were a significantly higher number of secondary follicles in the treatment group compared to the control (Table 13). The number of corpora lutea did not differ

between the control and treatment groups. The size of the ovary did not differ between the control and treatment groups (Table 14).

Table 13: Table showing the number of follicles within the ovaries of control and treatment group

Follicle type	Control N=16	Treatment N=11
Primary	4.44 ± 0.894	4.55 ± 0.779
Secondary	4.25 ± 0.602	7.09 ± 1.013*
Antral	1.3 ± 0.299	1 ± 0.330
Corpus luteum	1.9 ± 0.277	0.73 ± 0.237

Table 13 shows the average number of different types of follicles within the ovaries after 20 days administration of acetaminophen at 200mg/Kg. The results show a significant increase in the number of secondary follicles compared to the control. Mean follicle values are expressed as Mean ± SEM (N=5/ group, n=2). *P<0.05.

Table 14: Table showing differences in the size of the ovary in μm

Ovary	Control	Treatment
Length	1386.67 ± 129.97	1480 ± 114.08
Width	1003.33 ± 117.99	825.0 ± 88.91

Table 14 shows the size of the ovaries after 20 days administration of acetaminophen at 200mg/Kg. The results were not significant compared to the control. Mean length and width values are expressed as Mean ± SEM (N=5/ group, n=3).

Histologically, all types of follicles were visible in the control group but some stages were distinctly missing from the treatment group (Figure 13A: B). There was an increase in the number of atretic follicles in the treatment group compared to the control group. Follicles at various stages showed degenerative changes following the administration of acetaminophen for twenty days. There was the presence of degenerating antral follicles in the treatment group compared to the control (Figures 14A and B). The cytoplasm was condensed and there was a

shrunken ooplasm, a disrupted arrangement of granulosa cells, and a disruption in the zona pellucida (Figure 14B) compared to the control (Fig 14A). In the primary follicles, the theca cell layer was disrupted. However, the most significant degenerative changes were observed within the secondary follicles of the treatment group. The secondary follicles in the control group had a well-demarcated granulosa cell layer, zona pellucida, and viable oocyte (Figure 14A). The treatment group was lacking an oocyte and zona pellucida (Fig 14B). Granulosa cells were condensed hence appearing as if there was loss of cytoplasm. The inner lining of the granulosa cell layer had pyknotic cells and the theca layer was disrupted compared to the control.

A B

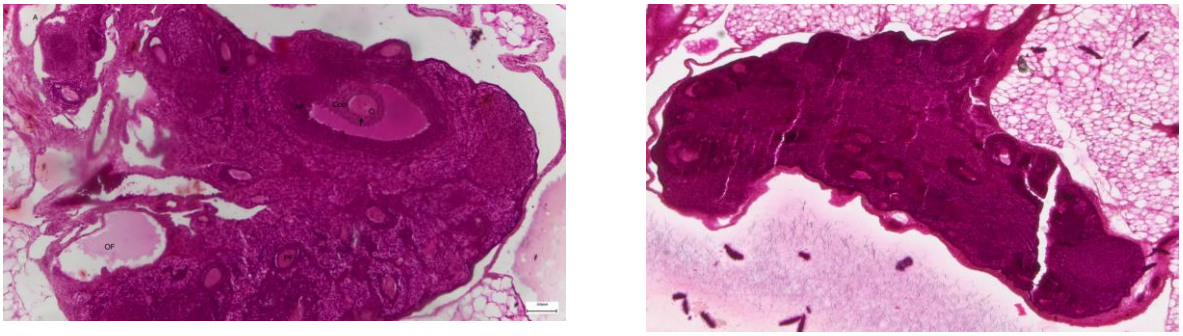

Figure 13: Effect of acetaminophen on the ovary.

Figure 13A control- photomicrograph showing the ovary with various stages of follicles. 13B shows an ovary from a treatment mouse with primary and secondary follicles. There is an absence of antral follicles.

A B

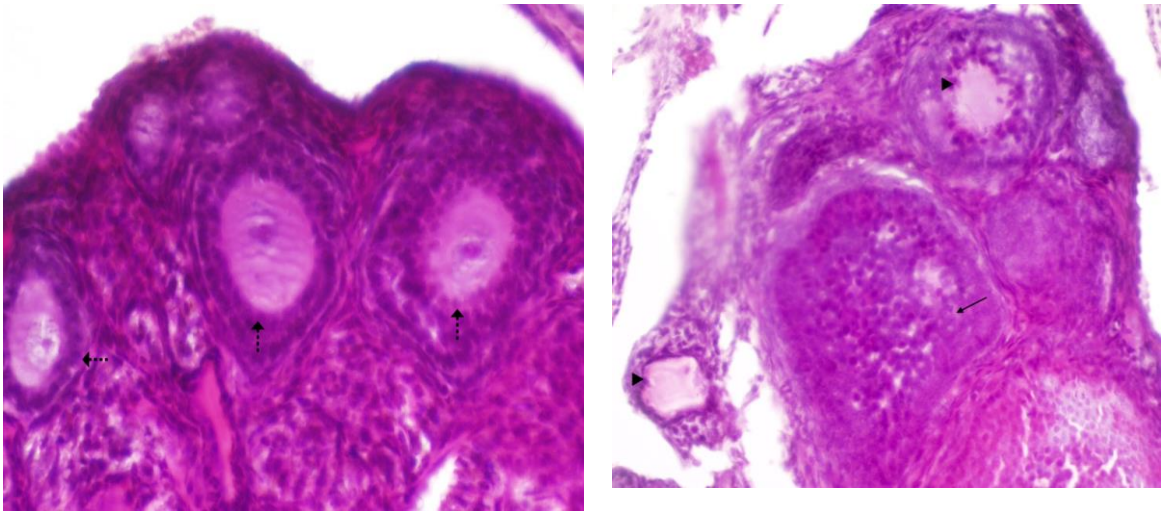

Figure 14: Effect of acetaminophen on the ovary.

14A: Control, the image shows secondary follicles (Black arrows). The structural integrity of granulosa cells is intact, there is the presence of viable oocyte and zona pellucida. 14B: Atretic secondary follicles, Granulosa cells (Black arrows) are condensed, there is lack of oocyte and zona pellucida (Black arrowheads).

A B

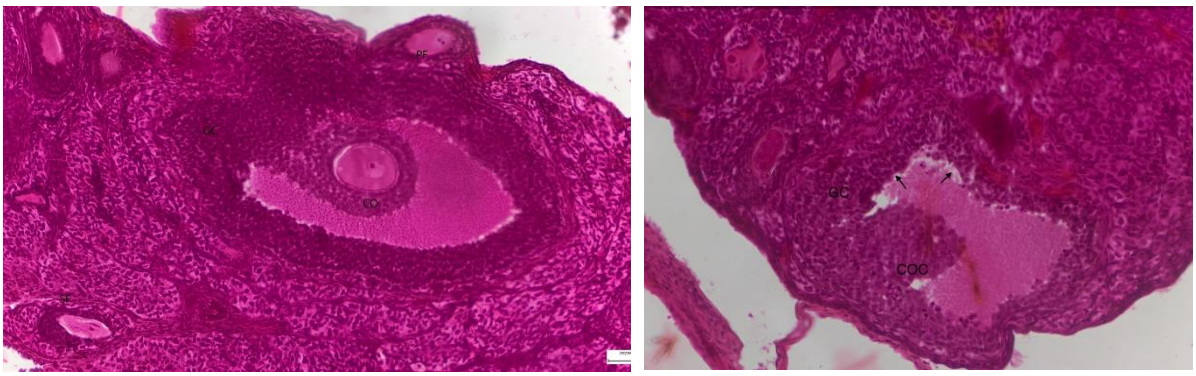

Figure 15: Effect of acetaminophen on the ovary.

15A: Control, the image shows an antral follicle. The structural integrity of granulosa cells is intact there is the presence of viable oocyte and zona pellucida. 15B: Atretic antral follicle lack of oocyte and zona pellucida. Granulosa cells condensed with loss of cytoplasm.

A B

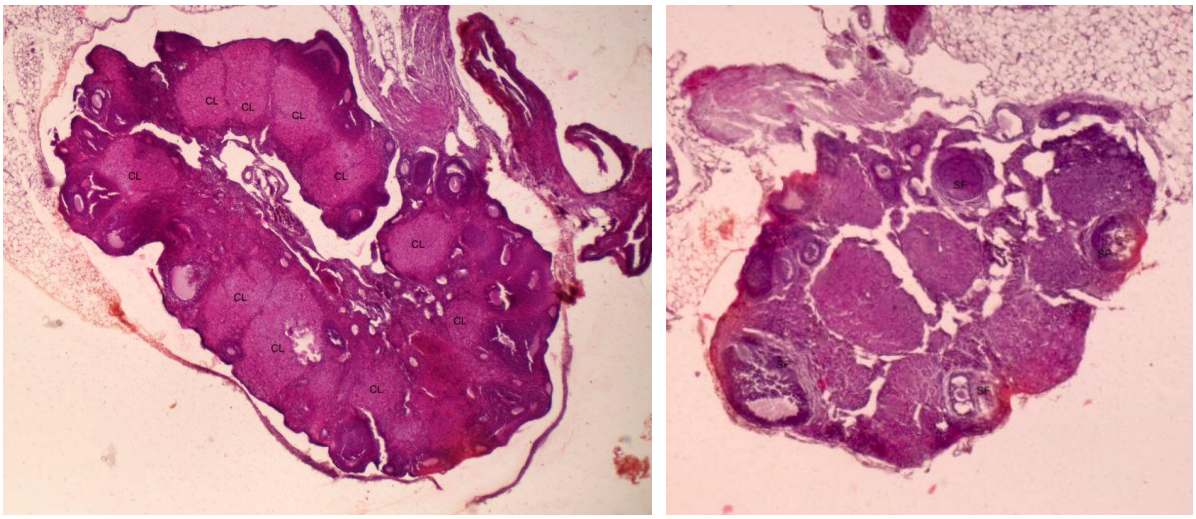

Figure 16: Effect of acetaminophen on the ovary

16A: Control- Photo showing corpora lutea (CL) with intact structural integrity. 16B: The Treatment - photomicrograph shows the presence of various stages of ovarian follicles. The number of corpora luteum was not significantly different from the control, however, the secondary follicles in 16B are undergoing atresia.

The thickness of the endometrium did not differ between the two groups. There was however an increase in the size of the endometrial glands in the treatment group. From observation, the glands in the control groups were many and small in diameter but those in the treatment group were fewer but larger in size (Figure 17 and Figure 18). There was a disruption in the endometrial lining in the treatment group compared to the continuous lining in the control group.

A B

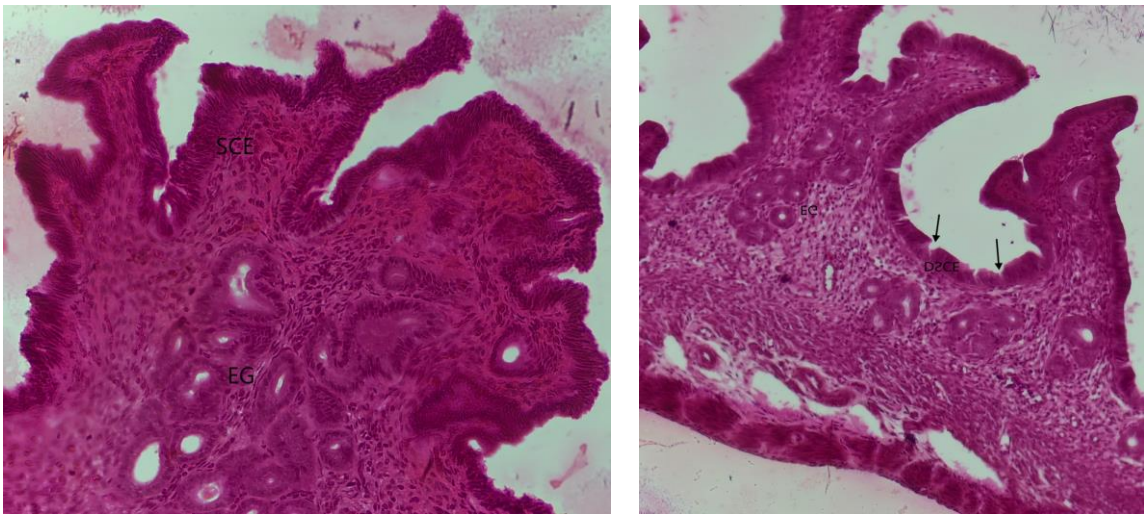

Figure 17: Effect of acetaminophen on the uterus.

Figure 17A: control showing uterus with continuous endometrial lining and numerous endometrial glands (EG). 17B shows endometrium from the treatment group in proestrus, there is disruption of the endometrial lining (Black arrows). Magnification X200

A B

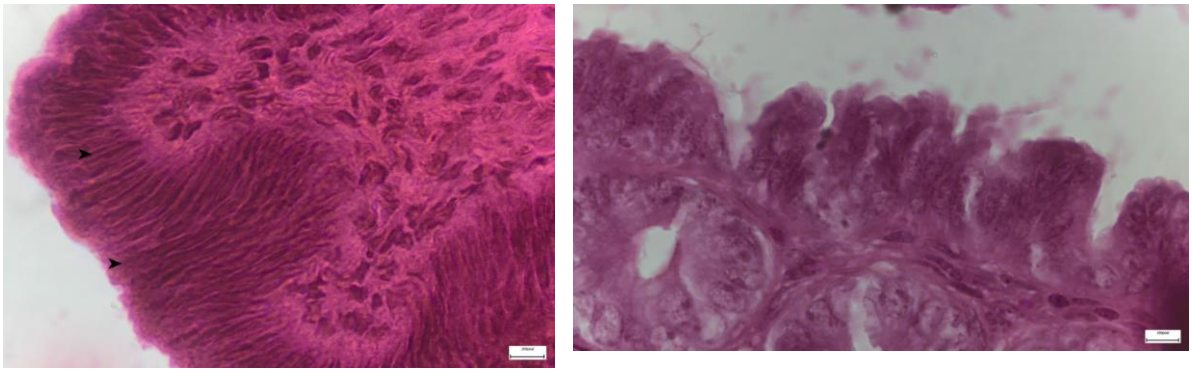

Figure 18: Effect of acetaminophen on the uterus

Figure 18A: Control- photomicrograph showing continuous endometrial layer compared to 18B- treatment showing disruptions in the endometrium. Magnification X100

6.4 DISCUSSION

There were a significantly higher number of secondary follicles in the treatment compared to the control group (Table 13). This high number could result from delayed transition into the antral follicle, which later ovulates, this could also signify a delay in ovulation and would explain the reduced number of implantation sites seen in chapter 4 of this study. This finding agrees with other studies done on NSAIDs such as Ibuprofen (Uhler *et al.*, 2001), meloxicam (Beta *et al.*, 2013) and celecoxib (Edelman *et al.*, 2013). From the studies Ibuprofen reduced the levels of cytokines and maternal metalloproteins (MMP) IL-6, IL-8 and G-CSF within the liquor folliculi resulting in delayed/inhibited ovulation (Mamata et al., 1992; Nemer et al., 2018). Granulosa cells produce IL-6, IL-8 and G-CSF (Mamata et al., 1992). IL-6 has proangiogenic properties and is involved in the formation of follicular vasculature. IL-8, on the other hand, works in concert with IL-6 to promote follicular rupture and the development of the corpus luteum. Therefore, it is likely that acetaminophen, through inhibition of prostaglandin synthesis, resulted in the reduction of these beneficial cytokines, which inhibited the growth of the secondary follicles and triggered atresia.

There were increased number of atretic secondary follicles in the treatment group. Pituitary gonadotropins have little effect on the recruitment, growth, or maturation of preantral follicles (Orisaka et al., 2021). FSH, on the other hand, is required to form the antral follicle (Gallot et al., 2012; Sonigo et al., 2018). Under the influence of LH, theca cells secrete androgens, which are transformed into oestradiol within granulosa cells as the antral follicle matures (Richards et al., 2018).

The zona pellucida (ZP) is a relatively thick extracellular coat surrounding the oocyte's plasma membrane (Kaingu et al., 2016). The ZP comprises three glycoproteins known as mZP1, mZP2 and mZP3, synthesised and secreted by the growing oocyte (Kaingu et al., 2016; Wassarman, 2008). The zona pellucida is laid down during the final stages of oogenesis when growing

oocytes enter their growth phase (Kaingu et al., 2016). The zona pellucida increase in diameter and thickness in concert with the oocyte diameter (15-80 μm). Kidder and Vanderhyden, (2010) have demonstrated that disruption of mZP1 results in a significant reduction of both secondary and tertiary follicles, consequently reducing the number of ovulated oocytes and litter size (Kaingu et al., 2016). In rats oocytes and ova lacking mZP2 and mZP3 glycoprotein also lack the zona pellucida and are likely to be infertile (Wang et al., 2020; Wassarman and Litscher, 2022). mZP2-/- and mZP3-/- mice do not develop the zona pellucida resulting in adverse effects on folliculogenesis causing a reduced number of antral follicles in ovaries and significantly lower numbers of ovulated ova in the oviducts (Liu et al., 1996).

Ovarian morphology from mZP2 and mZP3 -/- females gives the indication that developing and fully-developed oocytes are less closely linked with follicular and cumulus cells compared to oocytes from intact females (Kaingu et al., 2016). Particularly, cells of corona radiata from mZP2 and mZP3 -/- females are less orderly arrayed around growing oocytes than the control. A disruption of this cross-talk between oocyte and surrounding granulosa cells may be responsible for compromised folliculogenesis and oogenesis, especially where the structural integrity of granulosa cells and theca cells was compromised (Kaingu et al., 2016). Cumulus cells supply energy and nutrients including amino acids, glucose, and ribonucleosides to the oocyte through gap junctions (Gilchrist et al., 2008). Oocytes cannot metabolise glucose and can only generate ATP through oxidative phosphorylation (Warzych & Lipinska, 2020). However, cumulus cells consume glucose via aerobic glycolysis (Kaingu et al., 2016). Cumulus cells and the oocyte also communicate through paracrine signals. This signalling between the follicular cells and oocytes is bidirectional and vital for development (Kidder and Vanderhyden, 2010). A disruption in this paracrine signalling between mouse cumulus cells and their oocytes *in vitro* has been demonstrated to reduce oocyte competence (Kidder and Vanderhyden, 2010) and compromises fertility. This signalling may be crucial during pre-

ovulatory development, as the rate of pyruvate consumption in maturing metaphase-I oocytes is significantly higher than in immature oocytes (Kaingu et al., 2016). Cumulus cells also help the oocyte take in amino acids (Kaingu et al., 2016). As follicular cells are recruited in each oestrous cycle for growth and maturation, the oocytes grow and resume meiosis (Kaingu et al., 2016). This complex process involves a close interaction between the oocyte, surrounding granulosa and theca cells. In this study, the results in Figures 14B, and 15B, showed a disruption of the structural integrity of oocytes and surrounding cellular cells. This disruption suggests that the possible cause of infertility is compromised folliculogenesis and oogenesis (Kaingu et al., 2016).

There was impaired decidualisation of the endometrium in the treatment group. This impairment could be attributed to the high level of anandamide from acetaminophen intake. A high level of anandamide in the uterine lumen lowers the expression of COX-2 and VEGF. The high levels disrupt the decidualisation process (Fonseca et al., 2015). These uterine lumens also present with lower expression of NAPE-PLD and FAAH. Thus, anandamide is thought to directly inhibit decidualisation through actions on stromal cell differentiation within the reproductive tract system or indirectly by inhibiting COX-2-derived products and, subsequently, the vascular remodelling required for proper decidualisation.

The study showed no difference in the size of the ovary between the control and treatment groups (Table 14).

6.5 CONCLUSION

The study showed no difference in the number of primary and secondary follicles between the control and treatment groups. The size of the ovaries did not differ between the control and treatment groups. However, there was an increase in the number of secondary anovulatory follicles and a reduction in the number of corpora lutea in the treatment group.

CHAPTER 7

7.0 GENERAL DISCUSSION

Due of their short oestrous cycles and high levels of uterine anandamide, mice were used in this study (Paria *et al.,* 1996). In all experiments, there was no difference in the weight of mice between the experimental and control groups. This suggests that the 200 mg/kg dosage rate utilized in the study did not harm the mice. Greater than 300 mg/kg of acetaminophen has been associated with hepatotoxicity in mice. In addition, the mice resumed normal reproduction after the compound was withdrawn, indicating that it has the potential to be used as a contraceptive. A perfect contraceptive should have few negative effects and be reversible.

This study provides evidence linking acetaminophen's influence on ovulation and implantation to the regulation of female reproduction. Although acetaminophen has the ability to lower implantation sites and litter size, its usage is restricted to dogs due to its toxicity to cats. Acetaminophen is widely available without a prescription. At a dosage rate of 200 mg/kg, acetaminophen had no negative effect on the mice studied. This is a lesser amount than the recommended dose of 300 mg/kg. Therefore, it is suggested that additional research be conducted to determine the minimum effective dose.This study provides evidence linking acetaminophen's influence on ovulation and implantation to the regulation of female reproduction. Although acetaminophen has the ability to lower implantation sites and litter size, its usage is restricted to dogs due to its toxicity to cats. Acetaminophen is widely available without a prescription. At a dosage rate of 200 mg/kg, acetaminophen had no negative effect on the mice studied. This is a lesser amount than the recommended dose of 300 mg/kg. Therefore, it is suggested that additional research be conducted to determine the minimum effective dose.

Acetaminophen's effect on the female reproductive system has not been extensively studied. This study demonstrates that acetaminophen interrupts the oestrous cycle by significantly

decreasing the frequency of proestrus. In mice, acetaminophen negatively influenced reproduction. In this study, acetaminophen altered the pattern of FSH, LH, and Oestradiol production, resulting in reduced conception rates among the experimental animals. Together, pituitary gonadotropins and ovarian steroid hormones govern the oestrous cycle (Kaingu et al., 2016). The developing antral follicles begin producing and secreting oestradiol when the levels of FSH and LH reach a threshold level through synergistic action (Kaingu et al., 2016). Serum levels of both gonadotropins are regulated by ovarian steroids (Kaingu et al., 2016). The ovary produces oestradiol and progesterone in response to the LH hormone. This analysis revealed that the LH surge was delayed. As described in Chapter 4, a delayed oestradiol peak may have been the result of insufficient androgen production by theca cells (Kaingu et al., 2016). Problems with oestradiol production may have compromised endometrial priming and implantation in this study. Initiating and maintaining normal ovarian folliculogenesis necessitates an adequate quantity of FSH in the blood. Fertility is determined by the ability of the oocyte to be fertilized, develop a viable embryo, and have that embryo successfully implant itself (Kaingu et al., 2016). In this study, injection of acetaminophen after mating had the largest anti-fertility effect compared to the control (Table 10). As shown in Table 2, despite the possibility of a disruption in the oestrous cycle, mice in this study successfully mated. Successful mating would suggest that the mice's ovulation was delayed.

In this study, pre- and post-mating treatment of acetaminophen reduced the fertility index significantly. The poor implantation rates obtained in this study imply that acetaminophen has a deleterious effect on the developing embryo rather than halting its development. Oestradiol and progesterone are important hormones for preparing the endometrium for implantation (Kaingu et el., 2016). A disruption in sex hormones interacts with the uterine environment and has an effect on implantation (Kaingu et al., 2016). Progesterone hormone levels have an effect on the onset of pregnancy (Kaingu et al., 2016). In this study, progesterone levels were

considerably lower in the treatmennt group, which may have contributed to decreased decidualization and consequently fewer implant sites.

Every oestrous cycle recruits a group of developing follicles. Initiating and maintaining normal ovarian folliculogenesis requires an appropriate blood level of FSH. As FSH output gradually increases, oestradiol levels rise, initiating antral follicular growth and maturation. Rising oestradiol concentrations play a crucial impact in endometrial receptivity. After therapy with acetaminophen, the present histological and hormonal data suggest a malfunction of the hypothalamic-pituitary-gonadal axis. There may be fertility-regulating effects on the ovary, hypothalamus, and pituitary gland. As shown in the present study (Table 15), a delay in FSH synthesis can modify oestradiol profiles, resulting in an increase in atretic follicles, as described in chapter 5. As mentioned in Chapter 4, this would result in a considerable decrease in fertility index compared to the control group. It has been proven that the interruption of paracrine signaling between mouse oocytes and their cumulus cells in vitro lowers oocyte competence and affects fertility (Kidder and Vandenhyden, 2010; Kaingu et al., 2016). As the rate of pyruvate consumption in maturing metaphase-I oocytes is much higher than in immature oocytes, this signaling may be critical during pre-ovulatory development. Additionally, cumulus cells aid the oocyte in absorbing amino acids. As follicular cells are recruited for development and maturation throughout each oestrous cycle, the oocytes simultaneously grow and resume meiosis. The oocyte, surrounding granulula, and theca cells cooperate closely in this intricate process. In this study, Figures 11B and 12B revealed that the structural integrity of oocytes and surrounding cellular cells was compromised. According to these findings, infertility is the result of impaired folliculogenesis and oogenesis.

The exact mechanisms through which acetaminophen exerts this effect were not covered in this study and hence offer a chance for further research. Ovulation, fertilisation, and implantation are rate-limiting steps in the reproduction process that are targeted by most contraceptives in

use. A disruption in ovulation or/and implantation likely occurred, as shown by the significant reduction in the fertility index. This shows the potential of acetaminophen for use as a contraceptive.

These effects are reversible. After the withdrawal of acetaminophen treatment, the mice could conceive and give birth. This is an essential trait for any compound to be considered for contraception. It also shows that in case of infertility due to acetaminophen intake, it can be reversed by withdrawing usage. Therefore, women of childbearing age need to exercise caution when using acetaminophen.

This study did not show the molecular and biochemical effects of acetaminophen, which would help explain the research outcome. Further molecular studies are recommended to establish whether acetaminophen exerts these effects through inhibiting prostaglandin production or activating the endocannabinoid system.

7.1 GENERAL CONCLUSION AND RECOMMENDATIONS

7.1.1 GENERAL CONCLUSIONS

The study draws the following conclusions:-

1. Acetaminophen caused hormonal profile disruptions in treated mice; declined levels of progesterone and delayed peaks for FSH, LH and oestrogen
2. Acetaminophen significantly reduced both the number of implantation sites and the number of born kittens in treated mice
3. Acetaminophen treatment led to significant histopathology of both the follicles and the endometrium; increased number of degenerating secondary and tertiary follicles, and disruption of endometrium layer of the uterus

7.1.2 GENERAL RECOMMENDATIONS

From the observed conclusions the following recommendations can be advanced from this study:

- Acetaminophen has the potential in reducing number of neonates born from mice that are treated in both pre- and post-coital period during oestrus phase
- The observed pathophysiology points towards a mode of action of antifertility that speak to the advanced theory of acetaminophen acting through endocannabinoid systems (ECS) via its metabolite
- Further studies are required to fully elucidate the molecular entry point and active physiological routes of antifertility action of acetaminophen at cellular and organ levels

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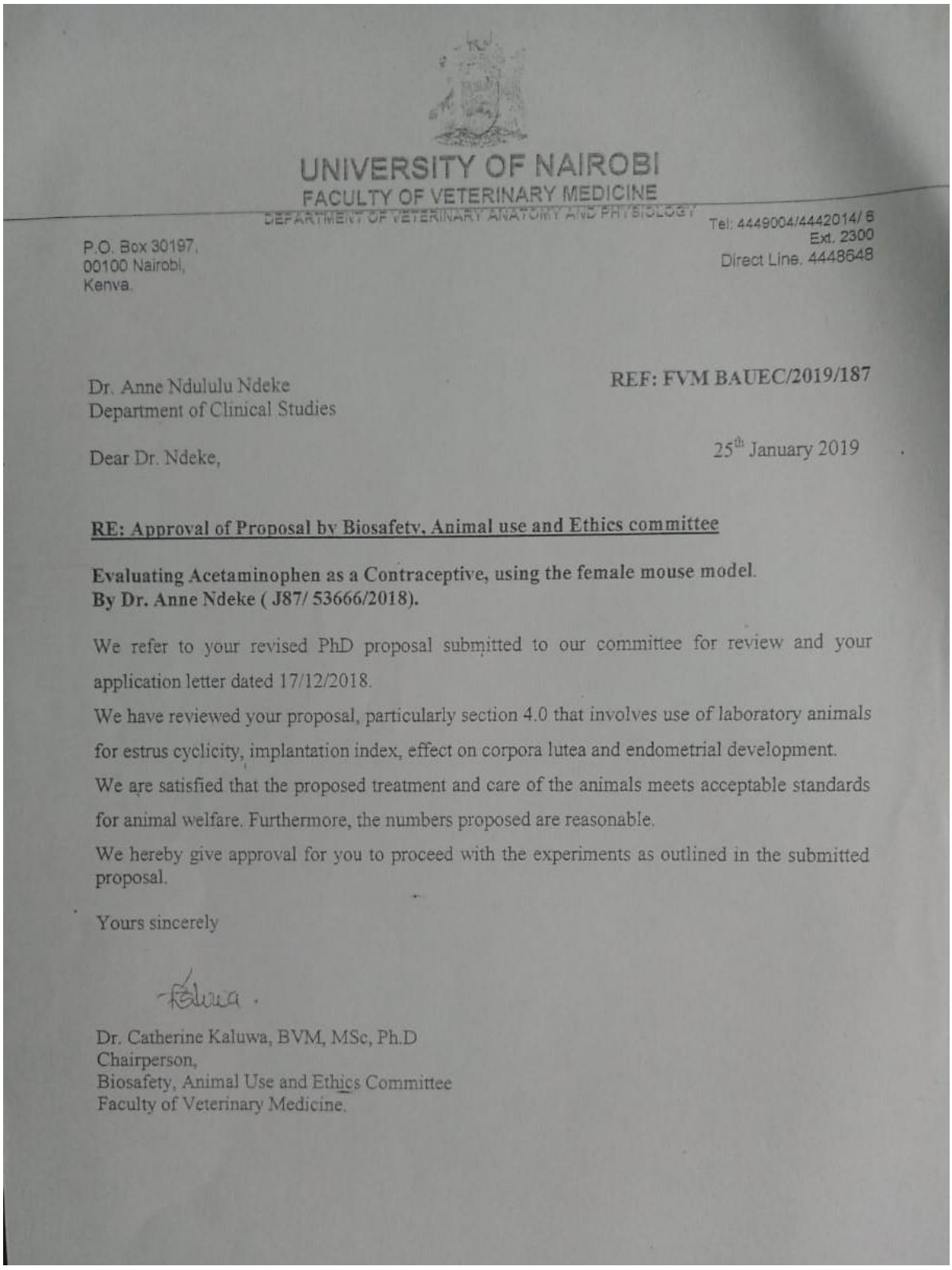
APPENDIX 1: BIOSAFETY, ANIMAL USE AND ETHICS CERTIFICATE



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Dr. Anne Ndululu Ndeke
Department of Clinical Studies

REF: FVM BAUEC/2019/187

Dear Dr. Ndeke,

25th January 2019

RE: Approval of Proposal by Biosafety, Animal use and Ethics committee

Evaluating Acetaminophen as a Contraceptive, using the female mouse model.
By Dr. Anne Ndeke (J87/ 53666/2018).

We refer to your revised PhD proposal submitted to our committee for review and your application letter dated 17/12/2018.

We have reviewed your proposal, particularly section 4.0 that involves use of laboratory animals for estrus cyclicity, implantation index, effect on corpora lutea and endometrial development.

We are satisfied that the proposed treatment and care of the animals meets acceptable standards for animal welfare. Furthermore, the numbers proposed are reasonable.

We hereby give approval for you to proceed with the experiments as outlined in the submitted proposal.

Yours sincerely

Dr. Catherine Kaluwa, BVM, MSc, Ph.D
Chairperson,
Biosafety, Animal Use and Ethics Committee
Faculty of Veterinary Medicine.

APPENDIX 2: EFFECT OF ACETAMINOPHEN ON THE OESTROUS CYCLE AND HORMONE PRODUCTION OF FEMALE MICE PAPER

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Effect of Acetaminophen on the Estrous Cycle and Reproductive Hormones of Female Mice

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ABSTRACT

In responding to the research question, what is the effect of acetaminophen on reproductive hormones and estrous cycles of mice? A study was undertaken to establish the potential impact of acetaminophen on the pituitary-gonadal axis. Reproductive hormones are critical drivers of regular estrous cycles in mammals. The hormones are required to achieve and maintain conception and the reproductive potential among domestic animal species. Up or down-regulation of any reproductive hormones interferes with the estrous cycle and may result in temporary or permanent infertility. Acetaminophen elicits analgesia through inhibition of prostaglandin synthesis and activation of the endocannabinoid system. This study aimed to investigate the effect of acetaminophen on the estrous cycle and reproductive hormone levels. The study utilized 6-8 weeks old female mice divided into control and treatment groups with five mice each. The control and treatment group received normal saline and 200mg/kg acetaminophen via oral gavage for 20 days, respectively. There was no difference in the length and number of cycles observed, but there was a significant reduction in the frequency of proestrus in the treatment group. There was a delay in producing estradiol, Luteinizing hormone and follicle-stimulating hormone in the treatment group. From this study, acetaminophen negatively affected the estrous cycle and hormone production in the treated mice. The observed disruption in hormone patterns could be a potential cause of infertility for both humans and animals that use acetaminophen.

Key words: Acetaminophen, Estrous cycle, Female hormones, Mice.

INTRODUCTION

Acetaminophen is readily available over the counter analgesic and anti-pyretic. Despite inhibiting cyclooxygenase (COX) production, acetaminophen is not considered as a typical non-steroidal anti-inflammatory drug (NSAID) due to its weak inflammatory properties. Due to this reason, there was limited research studies focused on its potential interference with reproduction.

The estrous cycle is a recurrent pattern of ovarian activity that enables a female animal to go from a period of reproductive receptiveness to non-receptivity with the failures. Non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to affect the female reproductive cycle causing reversible infertility (Stone et al. 2002).

Acetaminophen competitively inhibits prostaglandin synthesis. Acetaminophen competes with arachidonic acid for the Cyclo-oxygenase (COX) enzyme (Botting 2000). Oxidation of COX is necessary to exert its enzymatic activity (Sharma and Mehta 2014). Acetaminophen acts at the peroxidase (POX) site as a reducing co-substrate, indirectly interfering with this (Sharma and Mehta 2014). Acetaminophen stimulates prostaglandin synthesis at low concentrations and the reverse is true at high levels

APPENDIX 3: EFFECT OF ACETAMINOPHEN ON OVULATION AND IMPLANTATION IN FEMALE MICE PAPER

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Effect of Acetamınophen on Ovulation and Implantation in Female Mice

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ABSTRACT

Acetaminophen is known to inhibit prostaglandin synthesis and activate the endocannabinoid system, thereby has been linked to the regulation of mammalian reproductive processes through the same. Growing evidence tends to link acetaminophen reproductive effects in the regulation of ovulation and/or implantation. To provide further evidence, this study was designed to determine the effect of acetaminophen on ovulation and/or implantation. Female Swiss white mice were randomly divided into two experiments, one testing effects on ovulation and the other testing effects on implantation. The two groups were further sub-divided into treatment and control groups, each having N=5 mice. The mice in the treatment groups (TG1 and TG2) received 200mg/kg of acetaminophen while those in the control groups (CG1 and CG2) received the same quantities of normal saline. Mice in experiment group one (TG1, CG1) received acetaminophen/placebo before mating. Those in experiment group two (TG2, CG2) received acetaminophen/placebo for seven days post-mating. The presence of a vaginal plug confirmed mating success. The mice were sacrificed on the 7th day of pregnancy, the uterus harvested and all observed implantation sites counted and recorded. In both treatment groups (TG1 and TG2), a significant reduction in the number of implantation sites ($P \leq 0.05$) was observed when compared with the respective control group (CG1 and CG2). The observation points towards a role of acetaminophen in the regulation of ovulation and implantation in female mice reproduction.

Key words: Acetaminophen, Ovulation, Implantation, Mice, Reproduction.

INTRODUCTION

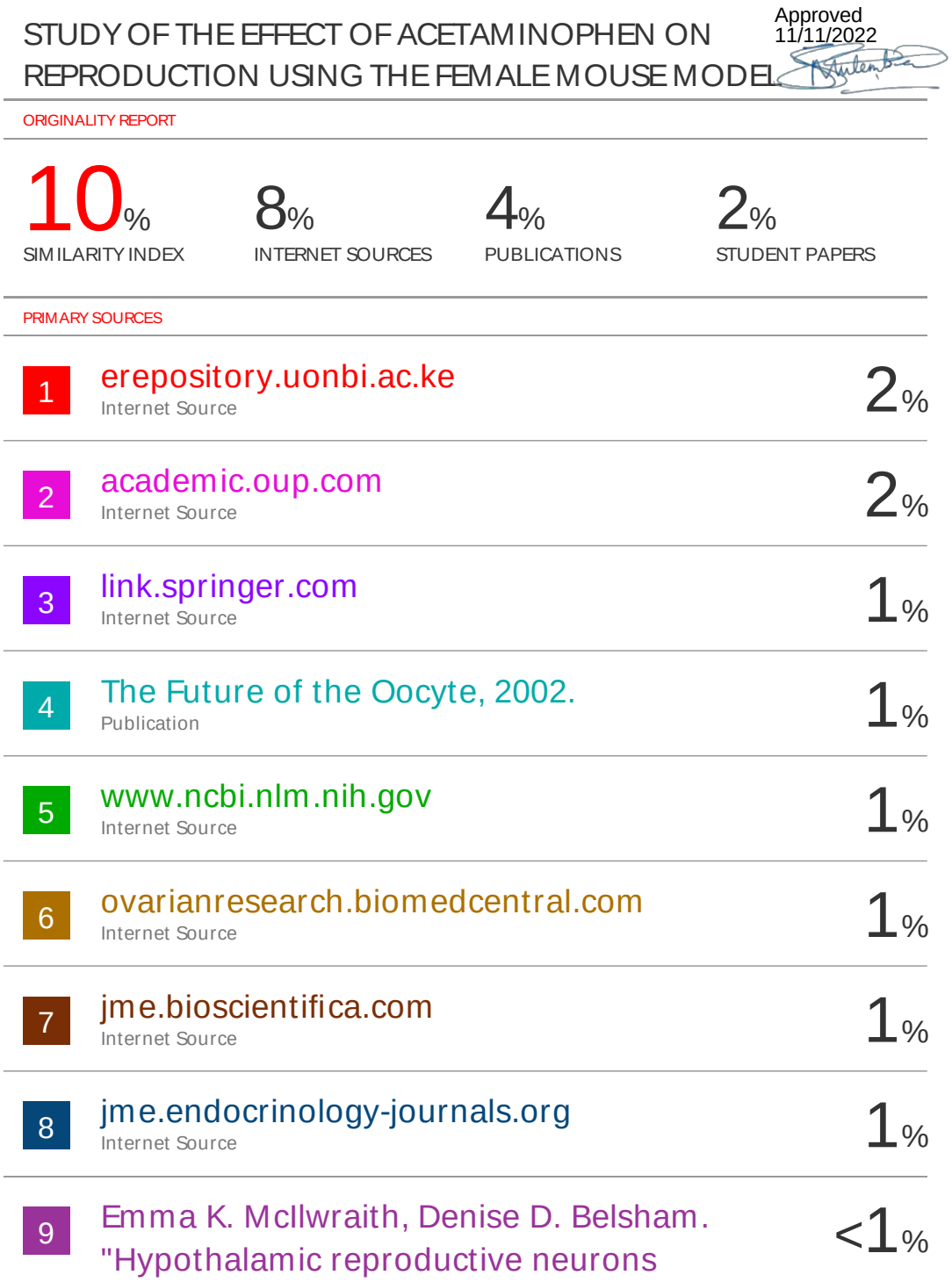
The Endocannabinoid System (ECS) has been shown to play a role in the regulation of mammalian reproductive processes through the synchronous crosstalk between the endometrium and the embryo to guarantee timely and successful implantation (Correa et al. 2016). Endocannabinoids that serve as ECS ligands are known to work through cannabinoid receptor type 1 (CB1) and 2 (CB2) and related enzymes, which have been demonstrated to be present in females (Cui et al. 2017; Cecconi et al. 2020).

Acetaminophen has been demonstrated to undergo metabolic deacetylation in the liver to yield *p*-aminophenol (Mazaleuskaya et al. 2015) that undergo further conjugation with arachidonic acid via fatty acid amide hydrolase (FAAH) to result in the formation of N-arachidonoyl-phenolamine (AM404) in the central nervous of anandamide. The inhibition of cellular uptake of anandamide leads to increased build-up in levels of the same (anandamide) within the extracellular space to regulate female reproductive processes probably at the levels of ovulation and/or implantation (Cui et al. 2017; Walker et al. 2019). This experiment was carried out to provide further clarification on the probable role of acetaminophen in the regulation of female reproduction using the mice as a model.

MATERIALS AND METHODS

All experimental procedures and ethical considerations on the use of animals were carried out as guided by the Kenyan animal welfare guidelines and approvals obtained from the Faculty of Veterinary Medicine University of Nairobi Biosafety, Animal Use,

APPENDIX 4: TURNITIN ORIGINALITY REPORT



STUDY OF THE EFFECT OF ACETAMINOPHEN ON REPRODUCTION USING THE FEMALE MOUSE MODEL

Approved 11/11/2022

ORIGINALITY REPORT

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APPENDIX 5: RESUME

OBJECTIVE

To improve the welfare of my immediate community by being a knowledgeable veterinarian with a positive attitude.

WORK EXPERIENCE

2019 to date: Tutorial fellow Theriogenology section, Department of clinical studies University of Nairobi

- Teach undergraduate BVM students' theory and practicals in Theriogenology
- Carry out clinical research

2017 - 2019: Branch Manager, Andys Veterinary Clinic Loresho. My responsibilities Included:

- Managing the branch's daily operation, i.e., administration and coordination of the branch's various departments, including the main clinic (organising the junior surgeons' duties), inventory, kennels, cattery, and nutrition management depending on the diagnosis of all admitted animals.

2013 - November 2017: Clinician, the Andys Veterinary clinic Limited- Loresho. My duties were:

- Animal handling and restraint
- Diseases diagnosis and treatment – Major on reproductive conditions.
- Sample collection, preservation, processing, and interpretation of results
- General pet care feeding, grooming and parasite control

2012: Project Facilitator- Impact of disaster interventions in Mwingi. My duties were:

- Meeting government veterinary officers, disaster management officers, local steering group members, and other delegates.
- Undertaking primary duties for the study, including translation and back translation of questions and the introduction and piloting of the survey.
- Carrying out participatory rural appraisals, key informant interviews and semi-structured interviews.
    - Aiding with other participatory methodologies within the study period, including mapping, transect walk, and direct observation.