INVESTIGATING THE EFFECTS OF TESTOSTERONE ON UTERINE FLUID REGULATION AND ENDOMETRIAL RECEPTIVITY IN A RAT MODEL

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THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

FACULTY OF MEDICINE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2016
UNIVERSITY OF MALAYA
ORIGINAL LITERARY WORK DECLARATION

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**ABSTRACT**

Normal regulation of uterine fluid volume, its ionic compositions and normal development of uterine receptivity are important factors that determine successful embryo implantation. The female sex hormones i.e. oestrogen and progesterone, are essential for the reproductive events, including sperm transport, capacitation, fertilization, embryo transport and implantation. In females, testosterone is produced in a very low amount by the ovaries and adrenal glands and is required for some of the normal physiological processes in the uterus. However, when present at the supra-physiological levels, such as in the pathological conditions examples polycystic ovarian disease and in excessive anabolic steroid intakes, the effects of this hormone may be detrimental for some of the physiological processes in the uterus. Up-to-date, effects of supra-physiological levels of testosterone on the regulation of uterine fluid and uterine receptivity have not been fully revealed. We hypothesized that supra-physiological levels of testosterone can affect these uterine parameters, thus could adversely affect the reproductive processes, which could compromise fertility. In this study, we have revealed that testosterone administration to ovariectomised rats, with or without 17β-estradiol decreased the uterine luminal fluid volume, chloride concentrations and cAMP levels and down-regulated CFTR expression but significantly enhanced the expression of Aqp-1, 5 and 7 in the uterus (p<0.05 when compared to in the absence of testosterone). Similarly, supra-physiological testosterone administration in sex-steroid replaced ovariectomised rats mimicking hormonal changes in early pregnancy reduced the pinopode development and MECA-79 expression, decreased the complexity of endometrial tight junction and down-regulated the expression of tight junction proteins i.e. claudin-4 and occludin. Additionally, the expression of αvβ3 integrin, E-cadherin and Muc-1 were also significantly reduced. The effects of testosterone in sex-steroid replaced ovariectomised rats were found to be mediated via the androgen receptor
however did not involve the active testosterone metabolites, DHT. Meanwhile, in the intact rats, testosterone administration from day 1 to day 3 of pregnancy suppressed pinopodes development, reduced the complexity of endometrial tight junctions and expressions of MECA-79, claudin-4, occludin, αvβ3 integrin, E-cadherin, Muc-1, Msx-1, Fgf-1 and Ihh proteins. Testosterone also reduced the number of embryo implantation. In conclusions, high levels of testosterone could adversely affect the female fertility via altering the normal regulation of uterine fluid volume, its chloride concentration as well as suppresses the endometrial receptivity development. These effects could suppress fertility.
ABSTRAK

ACKNOWLEDGEMENTS

In the name of Allah Most Compassionate and Most Merciful;

First, I would like to express my deepest gratitude to my supportive supervisor, Associate Prof Dr Naguib Salleh for his inspiring attitude, never-ending optimism and continuous encouragement throughout this work. Without his support and guidance, perhaps, I would not be able to complete this study. I would like to take this opportunity to express my deepest appreciation to Prof Dr Normadiah M Kassim, my supervisor for their continuous help and guidance during this study. I am indebted to both of them because guide me very well to reach a successful outcome. My kindest thanks belong to Prof Dr Sekaran Muniandy for their input, encouragement and give permission to me to perform molecular works in Protein Lab. Then, thank you very much to Dr Giri Babu for his guidance in research as well as moral support and advice during the period of my study.

Besides that, my gratitude goes to Prof Dr Ruby Husain, Head, Department of Physiology for her continuous support and also to all lecturers and staff of the Department of Physiology and Department of Anatomy that provide assistance throughout my study.

I would like to express my appreciation to Izzuddin Aziz, my housemate, UM-mate and friend since first degree for his assistance, support and motivation throughout this journey. For my friends- James, Riz, Khalil, Hariri, Nasrul, Hafiz, Nazery, Fazrul, Hairi, Muiz, Rais, Fira, Intan, Kamaliah, Ada, Kemala and others, thank you very much for being caring, encouraging and supportive friends and always have time to listen my problems and giving advice during difficulties in this challenging journey.
Besides that, it is my great pleasure to work with my lovely and helpful colleagues- Dr Khadijeh, Dr Firouzeh, Asma, Huma, Shin Yee, Su Yi, Ain, Aman, Suzanne, Bushra, Radwan, Hajar, Fatin, Ija and Dr Eliza. Thanks to all for being supportive and caring labmates instead of providing very friendly and excellent atmosphere in our lab.

I owe the greatest debt of gratitude to my beloved parents, Mokhtar Ujang and Faridah Ahamad for their compassion, support and love. Thank you very much for being a great father and mother to me. Then, a great thank to my brother; Saharul Mokhtar and sisters; Intan Zarina, Intan Nurliana, Nurul Ikmar and Nurasikin for your love, patience as well as the warmest support during my study period.

Last but not least, I would like to thank University Kebangsaan Malaysia for providing me a fellowship scheme and also to University Malaya for the research grant. Thanks to everyone that helped either directly or indirectly in completing my studies.
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SHBG</td>
<td>Sex hormone-binding globulin</td>
</tr>
<tr>
<td>T</td>
<td>Testosterone</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscope</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TJs</td>
<td>Tight junctions</td>
</tr>
<tr>
<td>TP</td>
<td>Testosterone propionate</td>
</tr>
<tr>
<td>WT-1</td>
<td>Wilms tumor</td>
</tr>
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</table>
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CHAPTER 1: INTRODUCTION

1.1 Research background

Testosterone (T) is a predominantly male sex hormone, which in females is produced by the ovaries and adrenal glands. The main circulating form of androgens in increasing order of abundance are dihydrotestosterone (DHT), T, androstenedione (A), dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS). DHT and T are capable of binding to androgen receptors (AR) while the remainder acts mainly as pro-hormones (Burger, 2002). T can both act directly on AR and being a pro-hormone for DHT and 17β-oestradiol (E) (Rathkopf & Scher, 2013). The presence of AR has been reported in the glandular epithelia and stroma of the endometrium, (Horie et al., 1992). Expression of stromal AR fluctuates with fluctuation in E and progesterone (P) levels (Bai et al., 2008). E was reported to up-regulate while P was reported to down-regulate AR mRNA expression in the uterus (Brenner et al., 2003). Additionally, AR expression was also been reported in decidua in the first trimester of pregnancy (Becker et al., 2011). This suggests that androgen was involved in maintaining early pregnancy.

T was reported to affect several uterine functions. In females, increased plasma T levels occur immediately following ovulation (Davison et al., 2005). T also plays an important role in embryo implantation (Cloke et al., 2008) where the decrease in its level below the physiological range can cause infertility (Krzeminski et al., 1998). Fluctuation in the plasma T levels occurs throughout the menstrual cycle, which is associated with changes in the expression levels of endometrial AR in glands and stroma (Horie et al., 1992). The level of AR in endometrium was reported to be up-regulated by E (Slayden & Brenner, 2004).
In diseases such as polycystic ovarian syndrome (PCOS), plasma levels of T were reported higher than normal (Apparao et al., 2002). Additionally, expression of AR in the uterus was also higher than normal (Apparao et al., 2002). PCOS-related infertility has been linked to the ovulation defects, however growing evidence suggested that high plasma T levels could suppress gene expression in the uterus for example WT1 genes that are involved in decidualisation (Gonzalez et al., 2012). High T levels were also able to reduce expression of cell apoptosis gene that participates in endometrial receptivity development (Yan et al., 2012).

Precise regulation of fluid in the uterus is crucial for successful embryo implantation. The volume and composition of uterine fluid were reported to be influenced by sex-steroids. E causes fluid accumulation that is important for many reproductive processes including sperm transport and capacitation, sperm-egg interaction, fertilization and embryo transport into the uterine lumen (Chan et al., 2002). Changes in uterine fluid volume and electrolyte compositions could result in infertility. Among factors that have been found to interfere with normal uterine fluid volume regulation include high doses E, anti-progestin and intrauterine contraceptive devices (Salleh et al., 2005).

Sex-steroid regulation of uterine fluid volume and electrolyte composition were found to involve ionic channels and membrane transporters such as aquaporin (Aqp) and cystic fibrosis transmembrane regulator (CFTR). Under E influence, Aqp-2 was reported to participate in fluid imbibition into the uterine lumen (Ehrmann, 2005). Meanwhile, P was reported to induce uterine fluid loss. P effects were shown to be mediated via Aqp-1 and 5 channels which expressions were up-regulated under this hormone influence (Gonzalez et al., 2012).

Embryo implantation starts with the blastocyst coming into contact with maternal endometrium. It represents the critical step of the reproductive process. Implantation
requires the embryo to develop into a competent stage and the endometrium to develop into a receptive stage. Uterine receptivity is a restricted period of time when the uterus is ready to accept the implanting blastocyst. The transition of the endometrium into the receptive state is coordinated by E and P (Paulson, 2011).

Development of uterine receptivity is associated with changes in endometrial morphology and expression of several proteins. The specific morphological changes during this period include the appearance of bleb-like projection known as pinopodes. Pinopodes can be seen at the apical surface of endometrial epithelium exclusively during the receptive period (Usadi et al., 2003). These structures are wide and extend above the microvilli. Pinopodes are transiently present and persist for 24 to 48 hours between days 19 and 21 of the menstrual cycle (Bentin-Ley, 2000). It is the site where the embryo first contact with the endometrium through its integral protein, the L-selectin ligand or MECA-79 (Bentin-Ley, 2000).

Besides pinopodes, expression of several proteins exclusively occurs during uterine receptive period, which include integrins, E-cadherins, L-selectins and mucin. Integrins are cell adhesion molecules involving in cell-matrix communications, which lead to cell migration and signal transduction (Lessey et al., 1994a). Three integrins are expressed which are α1β1, α4β1, and αvβ3. Integrins are one of the best-described markers of endometrial receptivity and expressed in both epithelium and stroma (Lessey et al., 1994a). Cadherins constitute a group of glycoproteins with thfunction that depends on calcium and mediates cell-to-cell adhesion. Cadherin is divided into 3 subclasses E-, P-, and N-cadherins. E-cadherin is the most studied subclass and is involved in the development of mouse embryos during pre-implantation period (Riethmacher et al., 1995). In humans, L-selectin expressed by trophoblast plays an important role in implantation (Smalley & Ley, 2005).
L-selectin ligand (MECA-79) is expressed on the pinopodes and mediates initial step in blastocyst-endometrial contact prior to blastocyst attachment (Foulk et al., 2007). Endometrial MECA-79 expression was reported higher in fertile than infertile humans (Margarit et al., 2009). Mucin-1 (Muc-1) is a high molecular weight glycosylated macromolecule that plays a role as an anti-adhesive agent. Muc-1 could interfere with cellular adhesion, therefore could play a role in preventing adhesion of the blastocyst to the endometrium. Tight junctions (TJs) and its integral proteins, claudin-4 and occludin serve as markers of uterine receptivity. TJs regulate passage of ions and molecules through the paracellular pathway and is important in preparing the endometrium for implantation (Burger, 2002).

I hypothesized that T affected fertility via interfering with embryo implantation. T effects can be mediated via disrupting the normal regulation of uterine fluid (volume and electrolyte compositions) and development of the uterus into the receptive state. A better understanding of the mechanisms underlying the effects of T on embryo implantation would provide an explanation for the high incidence of infertility in patients with high androgen level examples Polycystic Ovarian Syndrome (PCOS).
1.2 Significance of study

This study is important in order to elucidate the mechanisms underlying testosterone effects on uterine fluid volume and its ionic composition. This study also helps to elucidate the effects of this hormone on uterine receptivity development. By understanding detail mechanisms underlying testosterone effect on these parameters, the mechanisms that underlie fertility problems related to high plasma testosterone levels in females such as PCOS and excessive anabolic steroid intakes can be elucidated. Treatment to overcome the adverse testosterone effect on the uterus can then be implemented.

1.3 Objectives

This study utilized three models: (A) Ovariectomised + testosterone, with or without 17β-oestrogen treatment, to investigate the effects of testosterone on uterine fluid volume and ionic compositions. (B) Ovariectomised+sex-steroid replacement, with testosterone given during the receptivity period, to investigate the effects of testosterone on endometrial receptivity in ovariectomised rats receiving sex steroid replacement mimicking hormonal changes in early pregnancy. (C) Intact pregnant rats, with testosterone treatment during early pregnancy, to investigate the effects of testosterone on endometrial receptivity and embryo implantation in intact early pregnant rats.

The specific objectives of the study are:

To investigate the effects of testosterone on

i. uterine fluid secretion and chloride concentration.

ii. uterine ionic transporters that are involved in fluid regulation including cystic fibrosis transmembrane regulator (CFTR), aquaporin (Aqp)-1, 5 and 7.
iii. pinopodes and tight junctions development.
iv. the expression of uterine receptivity markers including MECA-79, αvβ3 integrin, E-cadherin, mucin-1.
v. the expression of tight junction proteins claudin-4 and occludin.
vi. the expression of signalling molecules such as muscle-segment homeobox-1 (Msx-1), fibroblast growth factor-1 (Fgf-1) and Indian hedgehog (Ihh).
vii. the rate of embryo implantation.
CHAPTER 2: LITERATURE REVIEW

2.1 Androgens

2.1.1 Background

Androgens are steroids produced by the ovaries and adrenal glands of females and are present at lower levels in pre-menopausal women compared to age-matched men (Zhang et al., 2004). The physiological effects of androgens are attributed to testosterone (T) which is the principal ligand for the androgen receptors and is the pro-hormone that can be converted to DHT and 17β-oestradiol (E) (Burger, 2002). In women, the major types of androgens that are listed in descending order based on its level in the serum are dehydroepiandrosterone sulphate (DHEAS), dehydroepiandrosterone (DHEA), androstenedione (A), T and DHT (Burger, 2002). The first three are considered as pro-androgens, which require conversion to T to express their androgenic effects. Only T and DHT have direct androgenic activities (Rathkopf & Scher, 2013).

High levels of T could be observed in the late follicular phase throughout the human menstrual cycle, as shown in Figure 2.1 (Sinha-Hikim et al., 1998; Rinaudo & Strauss, 2004). Plasma T levels also change throughout early pregnancy. In mice, its level reaches the peak at day 9 of pregnancy (Barkley et al., 1977). In parallel with changes in plasma T levels, the levels of androgen receptor (AR) also change throughout phases of the menstrual cycle (Bai et al., 2008) with AR mRNA was expressed in luminal and glandular epithelia, smooth muscles and stromal cells of the uterus (Pelletier et al., 2004).
**Figure 2.1:** Plasma testosterone levels in human females. This diagram illustrates the changes in endometrial histology and sex-hormone levels throughout the menstrual cycle.

Source: (Cloke & Christian, 2012)

### 2.1.2 Type of Androgens

#### 2.1.2.1 Testosterone

**Figure 2.2:** Structure of testosterone (T)
Testosterone (T) consists of 19-carbon steroids with the main sources in females being the theca cells and adrenal glands (Vermeulen, 1998). Biosynthesis of T in theca cells are controlled by luteinizing hormone (LH), which activates cyclic AMP (cAMP) that increases the transcription of genes for enzymes that are involved in the conversion of cholesterol to T. In adrenals, T is produced by zona fasciculata (25%) while in the ovaries, T is produced by the ovarian stroma (25%). Half of T in plasma is directly produced by these organs while the remaining 50% of circulating T are derived from the androstenedione (A) (Burger, 2002). In females, the daily production rate of T is between 0.1-0.4 mg/day with the circulating levels in the range of between 0.2-0.7 ng/ml. The lowest T concentrations were reported in the early follicular phase of the menstrual cycle and the levels rise in mid-cycle (Abraham, 1974). Removal of ovaries resulted in a fall of approximately 50% in T circulating levels (Miller et al., 2001).

The levels of T in the plasma of females could be affected by several factors. The plasma levels of total and free T was markedly reduced in women with hypopituitarism (Miller et al., 2001). Additionally, T levels fall slowly with age and did not change significantly during menopausal transition (Burger et al., 2000). T can undergo aromatization to E in female tissues that contain 17β-hydroxysteroid dehydrogenase and could also be converted to the potent androgen, DHT involving the 5α-reductase. T is metabolized mainly in the liver where it undergoes several biochemical reactions that are catalysed by different enzymes. Ultimate liver T metabolism results in T being converted to inactive compounds including androsterone and etiocholanolone (Henry & Norman, 2003).
2.1.2.2 Dehydroepiandrosterone Sulphate

![Image of DHEA Sulfate (DHEAS)]

**Figure 2.3:** Structure of dehydroepiandrosterone sulphate (DHEAS)

Dehydroepiandrosterone sulphate (DHEAS) is a product of adrenal zona reticularis with daily production at a rate of 3.5-20 mg/day and its circulating concentration in the range of 1000-4000 ng/ml. DHEAS is an important source of androgen (Haning et al., 1991). Its secretion by the adrenal glands is mainly regulated by ACTH and could be influenced by prolactin, IGF-1, and E. The circulating levels of this hormone do not change significantly during the menstrual cycle and during menopausal transition (Burger et al., 2000). Clinical deficiency of DHEAS could be observed in Addison’s disease, E replacement, hypopituitarism, corticosteroid therapy and chronic illnesses.

2.1.2.3 Dehydroepiandrosterone

![Image of DHEA]

**Figure 2.4:** Structure of dehydroepiandrosterone (DHEA)
Dehydroepiandrosterone (DHEA) is a secretory product of adrenal zona reticularis. This organ contributes about 50% of DHEA production while another 20% is produced by the ovarian theca cells. The remaining 30% is produced intracellularly from a peripheral synthesis of DHEAS that is catalysed by steroid sulphatase (Longcope, 1986). The production rate of DHEA is around 6-8 mg/day and its circulating plasma levels in human females is between 1-10 ng/ml. Levels of DHEA is found to decrease with age (Burger, 2002).

2.1.2.4 Androstenedione

Figure 2.5: Structure of androstenedione (A)

Adrenal zona fasciculata contributes about 50% of androstenedione (A) while another 50% is produced by the ovarian stroma. In human females, the daily production rate of A is at 1.4-6.2 mg/day while the circulating levels are in the range of 0.5-2 ng/ml. Removal of ovaries resulted in approximately 30% decrease in the circulating A levels (Burger, 2002). A can be produced intracellularly from DHEAS via DHEA and its level increased in the mid-cycle of the menstrual cycle parallel with the mid cyclical rise in E levels (Abraham, 1974). Administration of exogenous corticosteroids and hypopituitarism could suppress the A levels (Abraham, 1974).
2.1.2.5 Dihydrotestosterone

Figure 2.6: Structure of dihydrotestosterone (DHT)

Dihydrotestosterone (DHT) is a product of peripheral T conversion and circulates at low levels in serum (Abraham, 1974). Conversion of T to DHT involves the 5α-reductase enzyme, which reduces the double bond in the T molecule through the addition of two hydrogen atoms. Only a small quantity of DHT is produced directly by the adrenal zona fasciculata. The daily production rates of DHT in human females are between 4.3-12.5 mg/day with its circulating levels approximately 0.02 ng/ml. DHT functions like other androgens, but differs from T since it is more potent and has a stronger affinity to the AR. T can be aromatized to E however DHT cannot be aromatized to E (Burger, 2002).

2.1.3 Biosynthesis and production rate of androgens

Two critical enzymes regulate androgen biosynthesis that occurs in adrenal and ovaries are cytochrome P450 SCC and P450 c17. P450 SCC catalyses cholesterol side-chain cleavage while P450 c17 catalyses 17-hydroxylation which are required for DHEA and A production from pregnenolone and P respectively (Clark et al., 1994). Other important enzymes include 3-hydroxy steroid dehydrogenase (3-HSD) that catalyses the conversion of pregnenolone to P and DHEA to A while, 17-hydroxy steroid dehydrogenase (17-HSD) catalyses conversion of A to T. The side-chain
cleavage enzyme and steroidogenic acute regulatory protein (StAR) are the rate limiting enzymes for steroid synthesis. ACTH and LH also involve in regulating androgen secretion in the adrenal glands and ovaries respectively. Additionally, in these tissues, paracrine and autocrine factors are also involved in regulating the secretion of androgen. 3-HSD, 17-HSD and aromatase are also reported to be present in liver, adipose tissue and skin that make them capable of converting androgen to E (Lin et al., 1995).

**Figure 2.7:** Steroidogenesis in ovaries, adrenal glands and peripheral tissues.

Source: (Na et al., 2007)

As shown in Figure 2.7, adrenal glands and ovaries are the two main organs that function to produce androgen in women. Adrenal glands are the source for 100% DHEAS and 50% DHEA. Meanwhile, 30% of DHEA is produced from the peripheral conversion of DHEAS while the ovaries secrete 20%. A is produced at much lower amounts compared to DHEA which is approximately 3-5 mg/day lesser than DHEA. An approximately equal amount of A is being secreted by the adrenal glands (50%) and ovaries (50%). DHEAS, DHEA and A form a large reservoir for T, DHT and E.
Conversion of DHEAS and DHEA to T can occur in numerous peripheral tissues such as skin, fat, liver, and urogenital systems (Burger, 2002).

About 80% of T is bound to sex-hormone binding globulin (SHBG), 19% is bound to albumin while approximately 1% is freely circulating in the blood stream. The free form has influenced androgenicity since SHBG bound at high affinity to androgens (Fernlund & Gershagen, 1990). Meanwhile, DHEAS, DHEA, and A which are bound to albumin easily dissociate and available for action in the tissues as they have a lower binding affinity to the sex hormones.

2.1.4 Androgens and female reproductive system

Androgen receptor (AR) is expressed in the female reproductive tissue in a regulated manner. This receptor is localized in the glandular epithelia and endometrial stroma with the highest expression could be seen in the proliferative phase. However, the levels are decreased in the secretory phase and are almost undetectable in the late secretory phase (Brenner et al., 2003). Besides, AR is also localized in the stromal compartment of decidua during the first trimester of pregnancy (Zang et al., 2008). The cyclical changes of endometrial AR expression occur as a results in fluctuation of E and P level (Cloke & Christian, 2012).

The uterus is capable to synthesize DHEA, A, T and DHT. Endometrial intracrinology and enzymes including 17β-HSD, 3β-HSD and 5α-reductase are responsible for the interconversion of the steroid in this tissue. The extragonadal conversion into T also contributes to the uterine T levels (Ito et al., 2002).

The levels of circulating T are not parallel to the uterine T levels (Cloke & Christian, 2012). The uterine T levels could be higher compared to those in plasma such as during
the secretory phase, in which the levels of uterine T are increased approximately 4-folds (Vermeulen-Meninres et al., 1988). The changes of T levels in uterine tissues did not correspond to the magnitude of changes in plasma T levels. This differs from E and P in which the plasma and uterine tissue levels of these hormones were parallel (Davison et al., 2005).

Androgens are reported to exert multiple effects on the female reproductive organs. These hormones are found to be involved in maintaining follicular health where administration of DHT to primates is found to reduce the number of granulosa cell apoptosis (Vendola et al., 1998). Additionally, in humans, ovarian apoptosis was found to decrease in the presence of DHT (Otala et al., 2004). In pigs, T or DHT treatment during the follicular phase resulted in an increased ovulation rate (Cardenas et al., 2002). A study by Yang (2006) showed that T treatment increased the number of secondary follicles in ovaries with this effect is being antagonized by flutamide, a potent androgen receptor blocker. On the other hands, androgens can produce adverse effects on fertility whereby DHT administration in rats is reported to cause decreased in ovarian weight and the number of oocytes collected from the oviducts (Tyndall et al., 2012). Ovariectomised cows and mares treated with T showed decreased LH secretion by 17 to 26% (Thompson et al., 1984). Administration of T in intact rats prevented ovulation by blocking the LH surge while exogenous T administration in gilts increases levels of FSH (Jimenez et al., 2008).

T was reported to exert several effects on the uterus. Since the ovariectomised rat lacked uterine aromatase, androgen could not be aromatized to E (Bulun et al., 1993). In-vitro studies reported that androgen could inhibit endometrial proliferation (Tuckerman et al., 2000). Uterus expresses the AR which in cycling gilts are reported to be distributed in luminal and glandular epithelia of (Cárdenas & Pope, 2005). Similarly,
luminal and glandular epithelial distribution of the AR is also observed in humans (Kimura et al., 1993), immature rats (Weihua et al., 2002) and dogs (Vermeirsch et al., 2002) endometrium. The existence of AR in uterus indicates that T plays an important role in regulating the normal physiological function of this organ.

Table 2.1: Summary effects of androgen on female reproductive system

<table>
<thead>
<tr>
<th>No</th>
<th>Species</th>
<th>Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Human</td>
<td>Infertility and miscarriage - adverse effect of high androgen levels on the endometrium</td>
<td>(Tuckerman et al., 2000)</td>
</tr>
<tr>
<td>2</td>
<td>Human</td>
<td>Androgens possess antiproliferative effect on the endometrium - indicates that androgen inhibits cell growth and DNA synthesis</td>
<td>(Kalantaridou et al., 2006)</td>
</tr>
<tr>
<td>3</td>
<td>Mice</td>
<td>AR functions in the rat uterus as a modulator of myometrial and endometrial growth</td>
<td>(Nantermet et al., 2005)</td>
</tr>
<tr>
<td>4</td>
<td>Rat</td>
<td>Androgens appear to play a role in regulating cervical resistance by altering the proteoglycan content.</td>
<td>(Ji et al., 2008)</td>
</tr>
<tr>
<td>5</td>
<td>Human</td>
<td>Androgens induce relaxation of contractile activity in pregnant human myometrium at term</td>
<td>(Perusquia et al., 2005)</td>
</tr>
<tr>
<td>6</td>
<td>Pig</td>
<td>Androgens serve as substrates for the production of E by peri-implantation conceptuses and modulate the biological effects of E in the endometrium at peri-implantation.</td>
<td>(Kowalski et al., 2004)</td>
</tr>
<tr>
<td>7</td>
<td>Mice</td>
<td>Androgens enhance recruitment of primordial follicles into the growth pool and cause atresia of late antral follicles.</td>
<td>(Cheng et al., 2002)</td>
</tr>
<tr>
<td>8</td>
<td>Human and macaque</td>
<td>AR as a functional component of the mechanism in P antagonists induce endometrial antiproliferative effects in the presence of E</td>
<td>(Brenner et al., 2003)</td>
</tr>
<tr>
<td>9</td>
<td>Mice</td>
<td>T, A, and DHT can stimulate follicle growth and development</td>
<td>(Wang et al., 2001)</td>
</tr>
<tr>
<td>10</td>
<td>Rats</td>
<td>T associated with E induced metaplasia of the genital epithelium but not inhibit E induces metaplasia of the cervical and endometrial epithelium.</td>
<td>(Ramos et al., 2007)</td>
</tr>
<tr>
<td>11</td>
<td>Human</td>
<td>Increase level of DHEA induces detrimental effects on endometrial response resulting in miscarriage</td>
<td>(Sir-Petermann et al., 2002)</td>
</tr>
<tr>
<td>12</td>
<td>Rats</td>
<td>T treatment during the full postnatal life resulted in anovulation in adults with greater ovarian stromal compartment, and reduced numbers of anti-Mullerian hormone-positive smA follicles.</td>
<td>(Tyndall et al., 2012)</td>
</tr>
</tbody>
</table>
13. Rats  
T administration to immature female rats can induce polycystic ovaries, block ovulation and attenuate P production  
(Beloosesky et al., 2004)

14. Mice  
A low dose of TP led to a delay in embryo implantation and high doses of TP may disturb peri-implantation development or may be involved in early pregnancy loss by disturbing the uterine prostaglandin system  
(Diao et al., 2008)

15. Human  
The short-term treatment with T of postmenopausal women does not stimulate endometrial proliferation with T appears to counteract endometrial proliferation induced by E to a certain extent.  
(Zang et al., 2007)

16. Human  
T is involved in the regulation of sex hormone receptor expression in the postmenopausal endometrium and may therefore influence endometrial proliferation and differentiation.  
(Zang et al., 2008)

17. Human cell culture  
T inhibits the secretion and production of MMP-1 in HESCs in vitro, which indicate that androgen plays an important role in cyclic degradation and renewal of human endometrium.  
(Ishikawa et al., 2007)
2.2 Female reproductive system

2.2.1 Uterus

Uterus is a unique organ that supports the growth of embryo to full-term fetus. It is hormonal responses in which hormones are involved in preparing the uterus to provide the optimum environment for embryo implantation and for pregnancy support (Fritz & Speroff, 2011). Additionally, uterus also provides forceful contraction that aid delivery of the fetus. It remains in a relatively quiescent state in pre-pubertal and post-menopausal periods (Yen et al., 2014).

The human uterus shaped, resembling an upside down pear and consists of a thick muscular wall. Uterus consists of three parts including fundus, body and cervix (Niederer et al., 2009). This organ is located near the floor of pelvic cavity. However, in rodents, uterus comprised of two horns bounded by the cervix and vagina. The duplex structure of uterus allows implantation of multiple embryos (Suckow et al., 2006). In rats, mice and humans, blastocyst invades into the uterine wall and continues to grow to term.

The uterine wall consists of three layers known as perimetrium, myometrium and endometrium. Perimetrium is the outermost layer that provides external protection for the uterus. It consists of serous membrane, continuous with the abdominal and pelvic peritoneum that covers major organs of the abdominal-pelvic cavity. Perimetrium consists of a smooth layer of simple squamous epithelium that functions to protect the uterus from friction by secreting serous fluid to lubricate its surface. The myometrium forms the middle layer of the uterus and comprised of two layers of smooth muscles, inner circular and outer longitudinal muscles separated by connective tissue and blood vessels. During pregnancy, myometrium relaxes while during childbirth, it contractility increases to a maximum.
The innermost layer of the uterus is the endometrium, which is the most active layer that responds to the cyclical changes in ovarian hormones. Endometrium consists of connective tissue, stroma and uterine glands lined by simple columnar epithelium that continuous with the luminal epithelium (Crankshaw, 2001). Morphologically, the endometrium can be divided into functionalis and basalis layers. Functionalis layer made up the upper two-third of endometrium where dramatic changes occur in this layer during the menstrual cycle. Meanwhile, basalis layer undergoes a minimum change during the menstrual cycle and does not shed during menstruation. The endometrium exhibits proliferative and secretory changes during the menstrual cycle.

2.2.2 Reproductive cycle

Mammalian females experience regular cyclical changes in the endometrium in preparation for fertilization and pregnancy. In humans and primates, this cycle is known as the menstrual cycle while in rodent and other mammals, this cycle is called an oestrus cycle. Menstrual/oestrus cycle involves a series of changes under the control of ovarian sex hormones.

2.2.2.1 Oestrus cycle

In rodents and mammals except humans and primates, the reproductive cycle is known as oestrus cycle. This cycle represents the period from the onset of oestrus until the onset of the next oestrus. The length of the cycle varies with an average length in rats between 4 -5 days. The cycle consists of 4 phases namely prooestrus, oestrus, metoestrus and dioestrus (Long & Evans, 1922). These phases occur in a sequential manner. The oestrus cycle can be divided into two ovarian phases: follicular phase and
luteal phase. The follicular phase is a period where the ovarian follicles develop and consists of proestrus and oestrus phases. Meanwhile, the luteal phase is a period when corpus luteum is formed and comprises of metoestrus and dioestrus. Proestrus is a phase where the animal is coming into heat while metoestrus represents a phase of the absence of conception. Dioestrus is a phase where the uterus prepares to receive the competent blastocyst and anoestrus is the non-breeding season when reproductive organ is not receptive (Freeman, 1994).

The four phases of oestrus cycle are characterized by morphological and biochemical changes. These phases are best determined by distinct histology of the vaginal epithelium (Long & Evans, 1922). In order to identify histological changes, vaginal smears are examined in the morning at 0800 to 0900 h. The smears attained from the proestrus showed the appearance of nucleated round epithelial cells of almost constant size. The phase lasts for 12 - 24 hours. Oestrus phase lasts about 25-27 hours and is marked by the presence of anucleated cornified cell in the vaginal smear (Marcondes et al., 2002; Hubscher et al., 2005). Metoestrus stage lasts for 6 - 8 hours with the vaginal smear showed leucocytes and both cornified and nucleated epithelial cells in proportion. Dioestrus is the longest phase that lasts for 55 - 57 hours, which consists predominantly of leucocytes in the smear.

In rats, ovulation occurs spontaneously and copulation is needed to produce the functional corpus luteum. If mating does not occur, the cycle proceed to become the follicular phase however, if sterile mating occurs, pseudo pregnancy begins and this could last for two weeks. Vaginal plug can be observed in the following morning if rats mate during the oestrus phase. This plug remains for 24 hours (Long & Evans, 1922). The uterus undergoes hormonal changes during the oestrus cycle in which it is distended during proestrus and estrus stages because of increased in uterine vascular
2.2.2.2 Menstrual cycle

Menstrual cycle is a tightly coordinated cycle where changes occur in the ovaries and uterus, which are needed for ovum production and uterine preparation for pregnancy respectively. This cycle is governed by changes in the levels of ovarian E and P. In humans, a normal menstrual cycle lasts about 28 days with the length varies from 21-35 days (mean ± standard deviation of 28 ± 7 days) (Munster et al., 1992). The first day of menstrual bleeding is designated as day 1 of the cycle while ovulation occurs on day 14. The cycle can be divided into three main phases based on changes in the endometrium that correlated with functional changes in the ovaries. The endometrial phases include proliferative phase, secretory phase and menstrual phase (Buckley & Fox, 2002).

In proliferative phase, the endometrium regenerates and grows with the proliferation occur in the stroma and epithelia under the influence of E secreted by the developing follicles. During this phase, thickness of the endometrium increases with glands lined with stratified columnar cells. Additionally, oestrogen receptors (ER) α and β and progesterone receptors (PR) A and B are expressed in the endometrium (Chauchereau et al., 1992). This phase lasts for 11 days and ends at ovulation (Shier et al., 2002). The secretory phase occurs after ovulation and lasts for 12 days. This phase is related to an increase in P production by the corpus luteum in addition to E. In this phase, the endometrium is fully matured and undergoes secretory transformation under the combined effect of E and P. P induces development of secretory glands and enhances permeability. The increased in vascular permeability occurs in response to an accumulation of fluid under E stimulation (Becker et al., 2011). The distention starts to decline in late oestrus and it is no longer visible at dioestrus. The wet and dry uterine weights are heaviest at proestrus and lowest at dioestrus (Davison et al., 2005).
secretion of fluid, electrolytes and other molecules in the lumen of the uterine glands. During this phase, changes in endometrial provide an appropriate environment for the embryo to implant. Due to the high level of P in the mid-secretory phase, expression of ERα in glandular and luminal epithelia is down-regulated (Jabbour et al., 2006)

If no embryo implantation takes place in the secretory endometrium, the corpus luteum will degenerate followed by an abrupt decline in the circulating levels of E and P (Shier et al., 2002). The menses phase starts with degeneration and breakdown of the endometrium and also blood leakage into the uterine lumen. Menses last for 4-5 days and the endometrium will start to proliferate under the action of E. Figure 2.8 shows changes in hormone levels throughout human menstrual cycle and rat’s oestrous cycle.

![Graph A. Human Menstrual Cycle](image)

![Graph B. Rat Oestrous Cycle](image)

**Figure 2.8:** (A) Human reproductive cycle. (B) Rat reproductive cycle. A typical human reproductive menstrual cycle lasts 28 days, with ovulation occurs at mid-cycle on day 14 while the rat oestrous cycle is much shorter when compared to human cycle, and lasts 4 to 5 days.

Source : (Emanuele et al., 2002)
2.3 Implantation

Blastocyst implantation occurs within a short period known as the “window of implantation” (Giudice, 2006). Implantation window is defined as the limited time period when the uterus is ready to accept the blastocyst (Paria et al., 1993). The ovarian steroids including E and P regulate implantation (Carson et al., 2000; Paria et al., 2002; Dey et al., 2004). Communication between the blastocyst and the receptive uterus is crucial for successful implantation. Implantation can be divided into three phases: apposition, adhesion (attachment) and invasion (Carson et al., 2000). Following the invasion, trophoblasts invade into the endometrial stroma. Early pregnancy loss is associated with problems that occur prior to, during or after implantation in which the embryo is not competent or the uterus is not in the receptive state.

Fertilization occurs in the outer third of the fallopian tube 24 hours after ovulation (Speroff & Fritz, 2005). Following fusion of sperm and ovum, cell divisions occur and at the same time, the embryo moves down the fallopian tube towards the uterine cavity. The zygote undergoes mitotic divisions to form a morula, which is a cluster of blastomere. The morula then enters the uterine lumen, which then transformed into blastocyst stage that contains a cavity known as blastocoel. The blastocyst consists of two different types of cells, the inner cell mass and trophectoderm. Trophectoderm is the outer layer of the blastocyst that becomes the progenitor for the future trophoblast cells (Wang & Dey, 2006).

The blastocyst remains free floating within the uterine luminal fluid for 1–3 days before escapes from the zona pellucida and gain implantation competency (Spencer et al., 2004). The blastocysts further differentiate and possess three different cell types that include primitive endoderm, outer epithelial trophectoderm and inner cell mass. The
trophectoderm starts to make first physical contact with the endometrium to initiate the implantation process. Figure 2.9 shows sequential steps that occur during implantation.

![Embryo Implantation Diagram](image)

**Figure 2.9:** Sequential steps and cellular events during embryo implantation.

Source: (Glina et al., 2004)

### 2.3.1 Stages of implantation

#### 2.3.1.1 Apposition

In rats, the developing blastocyst enters the uterine cavity on day 5 of pregnancy. Once blastocyst hatches from zona pellucida, it is ready to initiate implantation with the first stage being apposition. Apposition is a transient and dynamic process. Uterine contraction and mucin, which lines the uterine lumen, involve in propelling the blastocyst within the cavity. These resulted in intimate but unstable contact between the trophectoderm and the receptive epithelium. Meanwhile, the loss of fluid in the uterine lumen at the time of blastocyst attachment was also thought to assist in bringing the blastocyst close to the luminal epithelium (Martin et al., 1970). Uterine closure sandwiches the blastocyst between the opposing uterine walls (Dey et al., 2004). The embryonic pole where the inner cell mass of the blastocyst are located makes the first contact with the receptive endometrium (Bentin-Ley & Lopata, 2000). Pinopodes are also thought to be involved in embryo apposition (Nejatbakhsh et al., 2012).
2.3.1.2 **Adhesion**

Paracrine signalling between the blastocyst and endometrium initiates a more stable adhesion following the establishment of apposition (Simon et al., 2000). Adhesion is associated with localized increase in stromal vascular permeability at the site of blastocyst attachment. Trophoblast surface interdigitates with the apical surface resulted in blastocyst closely attached to the endometrium as seen in Figure 2.10. The adhesion stage is regulated by cell adhesion molecules which include integrins, selectins, lectins and cadherins (Horwitz, 2012). The establishment of firm adhesion prevents blastocyst from being flushed away from the uterine cavity and in case it is forcefully removed, this will result in damage to the luminal epithelium (Wang & Dey, 2006).

**Figure 2.10:** Implantation stages of blastocyst into the endometrium
2.3.1.3 Invasion

In primates and rodents, the final step of implantation requires trophoblast to invade into maternal blood vessels leading to the formation of hemochorial placenta. The placenta serves as a site of fetal-maternal exchange during pregnancy (Gonzales et al., 1996). In mice and rats, the invasion process is initiated with apoptosis of the luminal epithelial cells. Following that, trophectoderm will invade between these cells and penetrate the basal lamina (Wang & Dey, 2006). Molecular interactions between the embryo and the endometrium are needed to secure normal invasion and survival of the embryo. Under the regulation of P, the endometrial stroma undergoes decidualisation where stromal cells and ECM transform into the decidua (Salleh & Giribabu, 2014). Decidua is an important structural and biochemical tissue that connects maternal tissue to the embryo. The implantation process completed when the blastocyst is embedded into the surface endometrium.

2.3.2 Uterine receptivity

Uterine receptivity is a state where the uterus is ready to receive the implanting embryo. This state occurs within a short period of time called window of implantation (Diedrich et al., 2007). Defective uterine receptivity may lead to implantation failure. Uterine receptivity can be divided into three phases, which include pre-receptive, receptive and refractory. In pre-receptive phase, the uterus is able to support embryonic development, but it is still not ready for implantation. The receptive phase represents the uterus is capable of initiating implantation when a competent blastocyst is present. Meanwhile, in refractory phase, the uterus is adverse to embryo attachment and subsequent implantation (Wang & Dey, 2006). In mice, the uterus is in pre-receptive phase from day 1 to day 3 of pregnancy. At day 4 of pregnancy, following E and P
priming, the uterus becomes fully receptive and ready to receive the competent blastocyst. In late day 5 of pregnancy, the uterus is hostile to blastocyst implantation. In human, the first 7 days of the secretory phase are referred as a pre-receptive phase. The receptive phase lasts for about 5 days, from days 7 - 11 after ovulation and the rest of the secretory phase represent the refractory phase (Paria et al., 1993; Wang & Dey, 2006).

Ovarian hormones play important roles in preparing the uterus for implantation. Steroid hormones synchronize rapid proliferation and differentiation of endometrial epithelial and stromal cells. In rats, E regulates epithelial cells to begin proliferation on days 1 to 3 of pregnancy while P, in combination with the E, initiate epithelial cell differentiation and stromal cell proliferation. By day 4 or 5, the epithelial and stromal cells are readily differentiated to receive the embryo for apposition, attachment and invasion processes. Besides that, ovarian hormones also affect the appearances of microvilli on the apical plasma membrane of uterine epithelial cells. E induces long, thin, regular microvilli while P induces short regular microvilli (Murphy, 1993). During the window of implantation, microvilli on the apical surface acquire flattened-shape before the arrival of competence blastocysts (Schlafke & Enders, 1975).

Uterine receptivity can be assessed by the presence of morphological and molecular markers. The specific morphological markers that have been proposed include pinopodes. Meanwhile, molecular markers associated with uterine receptivity include integrin, E-cadherin, mucin and L-selectins ligand (MECA-79). Synchronization of embryo development and expression of these molecules in the endometrium is important to achieve successful implantation (Figure: 2.11). Precise regulation of this process may help to prevent early pregnancy loss.
Figure 2.11: A schematic representation of blastocyst approaching the receptive endometrium. Early signalling between the blastocyst and the endometrium precedes attachment.

Source: (Staun-Ram & Shalev, 2005)

2.3.3 Markers of uterine receptivity

2.3.3.1 Pinopodes

In rodents and humans, pinopodes appear as a balloon or mushroom-like projections on the apical surface of the uterine luminal epithelium. The appearances of pinopodes can only be seen during the receptive phase. In mice and rats, pinopodes are thought to be involved in pinocytotic uptake of uterine fluid since it possesses many vacuoles (Parr & Parr, 1974). However, pinopodes lack membrane-bound organelles and are not covered by microvilli (Nikas, 1999). In humans, pinopodes also known as uterodome, are not pinocytotic (Adams et al., 2002). Human pinopodes contain Golgi complex, rough endoplasmic reticulum, secretory vesicles, mitochondria and nuclei except large
vacuoles (Adams et al., 2002). In rats, pinopodes can be found in approximately 20% of the epithelial cells, whereas, in humans, pinopodes are located only on the non-ciliated epithelial cells (Nikas et al., 1995). During uterine receptivity, three types of pinopode could be seen which include developing pinopodes, developed pinopodes and regressing pinopodes (Oborna et al., 2004).

Pinopode development starts with prominent cell bulging followed by the reduction in microvilli distribution (Nikas, 1999). The tips of microvilli fuse to form membrane projections that arise from the entire cell apex (developing pinopode). After a complete loss of microvilli, these projections achieve maximal size, folded and appear like a balloon or mushroom (fully developed pinopode). This stage lasts for less than 48 hours and the cell bulging starts to decrease with small tips of microvilli reappear on the membranes. The pinopode appearance is now wrinkled, and the cell size starts to increase (regressing pinopodes). Several molecules were reported to be involved in pinopode formation which includes leukemia inhibitory factor (LIF), HOXA10, αvβ3 integrin and Muc-1 (Quinn & Casper, 2009).

Pinopodes facilitate an intimate connection between the blastocyst and the endometrial surface. They are large enough to trap the cilia and prevent the blastocyst from being "swept away". Besides, pinopodes help to draw fluid and bring the developing blastocyst in close contact with the receptive epithelium prior to attachment (Bentin-Ley & Lopata, 2000; Oborna et al., 2004). The formation of pinopodes is strictly P-dependent, as administration of anti-progestin drug reduced pinopode expression (Petersen et al., 2005). E treatment also reduces expression of pinopodes (Martel et al., 1991). Since expression and life span of pinopodes are precisely controlled, this structure has been proposed as one of the best morphological markers of endometrial receptivity (Achache & Revel, 2006). In addition, a good correlation
between pinopode expression and periods of blastocyst hatching makes pinopodes as the best hint for the receptive period (Bentin-Ley et al., 1999).

The abundance of pinopodes correlates with successful implantation while less or fail pinopode development results in implantation failures (Nikas & Makrigiannakis, 2003). The number of pinopodes on the endometrial epithelium is important as developed pinopodes increases the contact area between the blastocyst and the endometrial surface. There are receptors for adhesion molecules on the pinopode surface, which are essential for embryo implantation.

2.3.3.2 Mucin

Mucin is glycoproteins that consist of two classes: secreted and transmembrane mucins. Mucin-1 (Muc-1) is a transmembrane mucin with a molecular mass of approximately 200 kDa. It is expressed on the apical surface of most epithelia, including the uterine luminal epithelium, mammary glands, lungs, kidneys, pancreas and in non-epithelial cells (Gendler & Spicer, 1995). Muc-1 spans up to 200 to 500 nm from the epithelial surface, a larger distance compared to other components of the apical glycocalyx (Hilkens et al., 1992). In the uterus, Muc-1 is abundantly expressed on the apical surface of both luminal and glandular epithelia (Julian et al., 2005).

Muc-1 possesses anti-adhesive characteristic. It plays a role as a barrier to microbes, proteolysis and implanting blastocysts. Muc-1 also lubricates and prevents dehydration of the cell surface and modulates cell-cell and cell-extracellular matrix interactions. During implantation, Muc-1 functions as a barrier along the apical surfaces to prevent blastocyst implantation during non-receptive phase (DeSouza et al., 1998; Brayman et al., 2006). The removal of this barrier is crucial to create embryo access to the apical
surface of uterine epithelium since the loss of Muc-1 was proposed to contribute to successful blastocyst attachment to the uterine epithelium (DeSouza et al., 1998). High levels of Muc-1 resulted in a refractory uterine endometrium in which the removal of this protein from the cell surface allows the blastocyst to attach (Chervenak & Illsley, 2000). High levels of Muc-1 can interfere with cellular adhesion via steric hindrance phenomenon (Fusani, 2008).

Ovarian steroids regulate the Muc-1 expression. In mice, E stimulated, while P down-regulated Muc-1 expression (Braga & Gendler, 1993; Surveyor et al., 1995). Meanwhile, in humans, Muc-1 expression is higher during the secretory phase, when P dominates. P upregulates the human Muc-1 expression (Hey et al., 1994; Meseguer et al., 2001).

In mice, expressions of Muc-1 dramatically decreased on day 4 of pregnancy, which represents the uterine receptive period (Surveyor et al., 1995). In rabbits, highest Muc-1 expression was observed during the receptive period where loss of Muc-1 expression was detected at the implantation sites (Hoffman et al., 1998). Similar patterns of expression can be seen in humans where highest Muc-1 expression was observed during implantation (Hey et al., 1994) and loss of Muc-1 only occur at the implantation sites (Meseguer et al., 2001). Levels of Muc-1 mRNA and protein increase several folds from proliferative phase to mid-secretory phase (Surveyor et al., 1995). It was suggested that the repellent effect of Muc-1 is important to guide blastocyst implantation. Significant reduction in Muc-1 expression can result in reduced rates of embryo implantation (Brayman et al., 2004).
2.3.3.3 E-cadherin

Cadherins are a group of glycoproteins responsible for Ca\(^{2+}\) dependent cell-to-cell adhesion. Based on its localization and immunological specificity, cadherins can be divided into E (epithelial), P (placental) and N (neural) cadherins. E-cadherin is a Ca\(^{2+}\) dependent adhesion molecule that regulates cell–cell adhesion and interactions as well as being the most studied subclass in the implantation process (Matsuzaki et al., 2010). E-cadherin expression can be observed in adherence junctions localized on the apical parts of the epithelial membrane.

E-cadherin is expressed in a variety of tissues (Tsuchiya et al., 2006) and plays a crucial role in embryogenesis (Barth et al., 1997). Down-regulation of E-cadherin expression could disturb the formation of cell–cell adhesion and pre-implantation embryo development (Riethmacher et al., 1995).

Loss of E-cadherin is associated with dissociation of cell-cell adhesion (Batlle et al., 2000; Cano et al., 2000). In addition, disturbance in intracellular Ca\(^{2+}\) concentration can affect adhesion of epithelial cells and its polarity. *In vitro* study showed that an increase in intracellular Ca\(^{2+}\) could down-regulate E-cadherin expression at the apical surface (Li et al., 2002).

The highest expression of E-cadherin could be seen in the luminal epithelium prior to implantation. However, this molecule is transiently down-regulated prior to blastocyst invasion into the stroma, indicating that remodelling of adhesion junction is critical during embryo implantation (Paria et al., 1999; Singh et al., 2000). There was evidence that a loosening of cell-cell junctions in uterine epithelium of mice via down-regulation of E-cadherin is a prerequisite for blastocyst attachment (Thie et al., 1996; Li et al., 2002).
Specific deletion of E-cadherin might results in implantation and decidualisation failures. The deficiency in adhesion and tight junctions in the uterine epithelium resulted in a disorganized cellular structure which fails to support embryo attachment and invasion (Reardon et al., 2012).

2.3.3.4 Integrins

Integrins are transmembrane glycoproteins that are formed by a combination of two different subunits, α and β (Achache & Revel, 2006). There are 18 α and 8 β chains in mammals. The combination of both subunits can form 24 integrin heterodimers that are distinct in their function, expression and localization (Hynes, 2002). These subunits consist of extracellular, transmembrane and intracellular domains. Integrins are involved in cell–matrix and cell–cell adhesion, essential for many physiological processes including embryo development, wound healing, immune and non-immune defense mechanisms and oncogenic transformation (Chan et al., 2002).

Binding of ligand determines the number and affinity of integrins that are present on the cell surface. This binding activates intracellular signal transduction pathway (Longhurst & Jennings, 1998). Different subunits of integrins are expressed in luminal and glandular epithelia of the endometrium. Integrins subunit expressed in the mid-luteal phase is proposed as a marker of endometrial receptivity (Lessey et al., 2000). α1β1, α4β1 and αvβ3 are three integrins subclass that is expressed in the endometrium with only αvβ3 integrin detected at the time of uterine receptivity (Lessey et al., 2000; Basak et al., 2002). αvβ3 is the first integrin found to interact with trophoblast (Apparao et al., 2001) and plays an important role as a potential receptor for embryo attachment (Lessey, 2003). The presence of integrins on both uterine epithelial surface and trophoblast mediate embryo adhesion.
The cycle-specific pattern of integrin expression is under hormonal influence. αvβ3 integrin expression in human endometrium is up-regulated by epidermal growth factor (EGF) and heparin-binding EGF (HB-EGF) but, down-regulated by E (Somkuti et al., 1997). During proliferative phase, high E levels inhibit the integrin expression via oestrogen receptor-α (ERα) (Achache & Revel, 2006). Meanwhile, increase in luteal P results in down-regulation of ER, thereby suppressing the inhibitory effects of E on integrins expression. P may provide a positive influence on the integrin expression in the endometrial epithelium by increasing the levels of paracrine factors in the stroma such as EGF and HB-EGF. These factors induce epithelial β3 integrin expression that serves as the rate-limiting step in αvβ3 formation (Lessey, 2003). Disturbance in αvβ3 integrin expression may be related to unexplained infertility (Tei et al., 2003), endometriosis (Lessey et al., 1994b), hydrosalpinx (Meyer et al., 1997), luteal phase deficiency (LPD) (Lessey et al., 1992) and polycystic ovarian syndrome (PCOS) (Apparao et al., 2002).

2.3.4 **Tight junction**

Tight junctions (TJs) are the most apical part of junctional complexes next to adherence junction (AJ). TJs represent a boundary in a region between plasma membrane of two adjacent cells that are closely positioned. TJs fuse cell together and provide important adhesive contact between neighbouring epithelial cells. It also forms distinct “seals” or “kissing point” at the apical portion of the lateral membrane between adjacent epithelial cells (Tsukita & Furuse, 2000a). The number of strands and interconnections determines the tightness of barrier formed by TJs network (Claude & Goodenough, 1973).
TJs regulate the diffusion of proteins and molecules by inhibiting free diffusion of molecules between apical and basolateral membranes and is involved in controlling ions, water and molecule transport across the paracellular space between the epithelial cells (Tsukita & Furuse, 2000b). Besides, the location of TJs at the apical membrane prevents entry of microorganisms and molecules from the apical portion of the epithelial cells (Turksen & Troy, 2004). At the time of implantation, TJs appear to tightly control the composition and volume of uterine luminal fluid (Murphy et al., 1982).

A freeze-fracture study by Murphy et al., (1982) in rats during early pregnancy and under treatment of different regimes of ovarian hormone showed that the TJs appearance in luminal epithelial cells is under hormonal control. The luminal epithelial cells under E dominance show parallel TJs network while under P effect, TJs appear geometrically complex, interconnected and extends down to the lateral plasma membrane (Orchard & Murphy, 2002).

The composition and ratio of transmembrane protein are factors that contribute to permeability and selectivity of TJs (Tsukita et al., 2001). These transmembrane proteins include claudin, a large family of transmembrane protein and occludin which is a transmembrane protein with several splice variants. Claudin is the key component of TJs that connect adjacent cells and apparently polymerize within the plasma membrane as fibrils to generate TJs strand (Furuse & Tsukita, 2006; Van Itallie & Anderson, 2006; Angelow et al., 2008). Meanwhile, occludin is a four transmembrane domain-containing proteins with related amino acid sequences that are incorporated and localized very close to claudin-based TJs strand (Furuse et al., 1993; Fujimoto, 1995).
2.3.4.1 Claudin

Claudins are 21-28 kD transmembrane proteins that comprise of four transmembrane domains, two extracellular loops, amino- and carboxy-terminal cytoplasmic domains and a short cytoplasmic turn. Claudins consist of multigene families with 24 members in humans and mice (Furuse & Tsukita, 2006; Angelow et al., 2008).

Claudins are one of the important proteins co-functioned with occludin to determine TJs selectivity. Claudins are pivotal components of the structure and function of TJs strands. Besides that, claudins also show Ca\textsuperscript{2+}-independent cell adhesion which is the major elements of the barrier function of TJs (Kubota et al., 1999). Claudins coordinate the gate function of paracellular TJs that have biophysical properties similar to those of traditional ion channel, including permeability which depends on ion concentration, ion charge selectivity and competition for movement of permeable molecules (Tang & Goodenough, 2003). Any changes in the type of claudin expressed may affect ion selectivity and may induce organ-specific defects.

Previous studies in a mouse lacking endogenous TJs showed that exogenous administration of claudin causes this protein to concentrate between adjacent cells and generate TJs strand (Kubota et al., 1999; Sasaki et al., 2003). On the other hand, removal of claudin from TJs reduced the number of TJs strand and decreased TJs barrier function (Sonoda et al., 1999).

Claudin 1, 3, 4 and 5 were found to be expressed in the uterus during oestrus cycle and in early pregnancy (Nicholson et al., 2010). Apart from that, claudin 3 and 4 show specific localization in the TJs with only claudin-4 showing strong expression at the time of implantation under influence of P (Carson et al., 2002). Additionally, claudin-4 could contribute to the increased complexity of TJs strand network and decreased the paracellular conductance (Nicholson et al., 2010).
2.3.4.2 Occludin

Occludin is a 65-kD integral membrane protein with four transmembrane domains, two extracellular loops and intracellular turn i.e. carboxy- and amino-terminal cytoplasmic domains. Occludin is the first identified protein of TJs that isolated from the liver of chicken (Furuse et al., 1993). It is an essential transmembrane in all epithelial and endothelial tissues and in neurons and astrocytes (Choi et al., 2012). The presence of occludin is important for establishing a paracellular barrier (Tsukita et al., 2001). The amount of tissue occludin is inversely proportional to the TJs permeability (Moon et al., 2007). Additionally, occludin is also crucial for uterine receptivity development. Expression of occludin is under the influence of P and can be detected in the uterus during the receptive period (Orchard & Murphy, 2002). However, under E influence, it is undetectable in the uterus.

Up-regulated expression of occludin increases the tightness of TJs (Qin et al., 2006). Occludin also communicates with claudin to regulate the paracellular permeability that controls the composition and volume of the uterine luminal fluid (Balda et al., 2000). Occludin is involved in the formation of an intramembranous diffusion barrier that prevents fusion of apical and basolateral membranes. In mice lacking fibroblasts, exogenous administration of occludin increases the number of TJs strands (McCarthy et al., 1996). Selective paracellular diffusion depends on the composition of occludin since it is the main component that regulates opening and closing of the paracellular pathway (Balda et al., 2000).
2.3.5 Endometrial signaling molecules

2.3.5.1 Muscle-segment homeobox

Muscle-segment homeobox (Msx) genes are one of the most conserved and oldest families of homeobox genes in animals (Finnerty et al., 2009). It is associated with the Drosophila ms gene (Cornell & Ohlen, 2000) and acts as downstream targets of bone morphogenic protein (BMPs) during development (Bei & Maas, 1998; Timmer et al., 2002). In mice, Msx family consists of three different genes, Msx-1, Msx-2 and Msx-3 (Davidson, 1995). Previous studies showed that Msx-1 acts as a negative regulator of differentiation (Woloshin et al., 1995). Additionally, Msx-1 and Msx-2 act as transcriptional repressors (Zhang et al., 1996; Newberry et al., 1997). However, less is known about Msx-3. Msx genes play an important role in determining epithelial cell polarity that is required for blastocyst attachment to the luminal epithelium. Loss of Msx expression alters the luminal epithelial integrity and apical-basal polarity where these changes could affect the formation of E-cadherin/β-catenin complex at adherens junctions via Wnt signalling. In the uterus, Msx-1 expression was reported in the luminal and glandular epithelia with the levels markedly reduced at day 4.5 of pregnancy coinciding with blastocyst implantation (Pavlova et al., 1994). Studies by Pavlova et al., (1994) showed that the uterine Msx1 expression requires LIF signalling and that is transient cell-specific expression around the time of implantation indicate its crucial role in implantation.
2.3.5.2 Fibroblast growth factor

Fibroblast Growth Factor (Fgf) is a family of multifunctional mitogenic polypeptides, which are part of the paracrine effectors of proliferation and angiogenesis in many tissues (Sak et al., 2013). Fgf-1 is known to be involved in promoting epithelial cell proliferation via E signalling and facilitating the establishment and maintenance of pregnancy in some mammals (Li et al., 2011).

2.3.5.3 Indian hedgehog

Mammalian Hedgehog proteins can be divided into three hedgehog genes, which include Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh) (Pathi et al., 2001). Hedgehog family play roles in mediating cellular processes from embryonic to adult in humans and many animal species (Walterhouse et al., 1999; Ingham & McMahon, 2001; Walterhouse et al., 2003). Ihh is expressed at high levels in uterine luminal and glandular epithelia prior to receptivity, but is down-regulated in the epithelium on receptive uterus at day 4 (Paria et al., 2001). It was reported that P effect on stromal cell proliferation is mediated via the paracrine action of Ihh secreted by the glandular epithelium (Wetendorf & DeMayo, 2012).
2.4 Uterine luminal fluid

2.4.1 Composition and Regulation

Uterine luminal fluid contains organic solutes, ions, and macromolecules such as Na\(^+\), K\(^+\), Cl\(^-\), Ca\(^{2+}\), H\(^+\), HCO\(_3^-\), glucose, amino acids and as well as proteins such as placental protein (Cloke et al., 2008). In uterus and oviduct, the fluids are alkaline, with pH varies from 7.6 to 8 depending on species (Chan et al., 2012). In rats, pigs and humans, the uterine luminal fluid was observed to contain high amounts of HCO\(_3^-\) and K\(^+\) with concentrations higher than that in plasma. Meanwhile, levels of Na\(^+\) in the uterine fluid are 20-30 mM lower than its level in plasma (Schultz et al., 1971; Matthews et al., 1998). Lower Ca\(^{2+}\) and Cl\(^-\) concentrations were recorded in human uterine fluid compared with their levels in the plasma (Casslen & Nilsson, 1984). The significant differences in the uterine fluid composition between uterine fluid and plasma indicate that the uterine epithelial layer actively transports ions and fluid into the uterine lumen and extracellular space.

Fluctuations in uterine fluid volume were observed during the oestrus cycle of rats, with the maximum fluid volume during the proestrus and oestrus while minimum fluid volume in dioestrus phase (Long & Evans, 1922). Reduction in the volume of fluid at metoestrus or dioestrus stages facilitates closure of the uterine lumen that assists embryo implantation. It was found that the uterine fluid consists of two to four folds higher bicarbonate content compared to plasma (Vishwakarma, 1962; Murdoch & White, 1968). Bicarbonate is needed for events such as sperm motility, capacitation and acrosomal reaction as well as for embryo implantation and development.

Precise regulation of uterine fluid environment is important for a number of reproductive events including sperm transport and capacitation, fertilization, embryo transport, development and blastocyst implantation (Chan et al., 2002). The ovarian
hormones play an important role in regulating the volume and compositions of uterine fluid (Bauersachs et al., 2005). E induces uterine luminal fluid accumulation, while P causes reduction of uterine fluid volume (Clemetson et al., 1977). Fluid accumulation is important for sperm and embryo transports. Mammalian sperms are able to fertilize the oocytes when they undergo capacitation (Chang, 1951). Meanwhile, fluid loss is vital to assist closure of the uterine lumen prior to the apposition and the adhesion phase of embryo implantation (Martin et al., 1970; Hu & Spencer, 2005). This could help to minimize the embryo movement and maintain close contact between the embryo and the uterine epithelium. Disturbances of the volume and composition of uterine luminal fluid could reduce the fertilization rate and might cause infertility.

The movements of fluid across luminal epithelia are secondary to the movement of ions. Na\(^+\) and Cl\(^-\) conductance are important to initiate fluid secretion as well as absorption. The epithelial lining of female reproductive tracts is able to absorb and secrete electrolytes. Active Na\(^+\) absorption can drive Cl\(^-\) counter ion, resulting in the withdrawal of fluid from the lumen into the blood vessel. Meanwhile, active Cl\(^-\) secretion can drive both fluid and Na\(^+\) from the blood into the uterine luminal.

### 2.4.2 Ion channels involved in fluid regulation

Ion channels are a group of transmembrane protein that allows movement of ions across cells, which could result in changes in the membrane potential, ion gradient and pH (Hille, 1986). These features make ion channels crucial for a number of physiological events such as cell volume regulation (Lambert et al., 2008), epithelial secretion and absorption (Chambers et al., 2007; Schild, 2010) and cell proliferation and apoptosis (Pardo, 2004).
Several ion channels have been identified to be expressed in the endometrial epithelium (Chan et al., 2012). These channels involved in regulating the ion transport that drives the water movement across the epithelium being either secretion or absorption (Salleh et al., 2005). Deviation from normal regulation of these ions could result in disordered fluid transport and implantation failure (He et al., 2010).

### 2.4.2.1 Cystic fibrosis transmembrane conductance regulator

Cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP-dependent anion channel, which belongs to the ATP-binding cassette family (Hwang & Kirk, 2013). As an apical Cl\(^-\) channel that is expressed in the reproductive tract, CFTR is involved in regulating Cl\(^-\) secretion and fluid movement into the lumen. Cl\(^-\) channels are rate limiting for Cl\(^-\) efflux and water movement. The importance of CFTR in uterine fluid regulation is highlighted in cystic fibrosis (CF), an autosomal recessive genetic disease caused by mutations of CFTR. CF caused defects in the electrolyte and fluid transport in almost all exocrine glands and tissues (Quinton, 1999; Rowe et al., 2005). It has been demonstrated that CFTR also mediates secretion of bicarbonate through the apical membrane of endometrial luminal epithelia as shown in Figure 2.12 (Wang et al., 2003; Chan et al., 2009).

CFTR was reported to be expressed in most epithelial lining, including the airways, gastrointestinal and reproductive tracts (Trezise & Buchwald, 1991). In the female reproductive tract, CFTR expression has been reported in the uterus, cervix, vagina and fallopian tubes (Tizzano et al., 1994; Chan et al., 2002). CFTR was localized to the apical membrane of the endometrial epithelium (Salleh et al., 2005).
E and P were reported to regulate CFTR expression in which E stimulates while P attenuated expression of this channel (Rochwerger & Buchwald, 1993; Rochwerger et al., 1994; Mularoni et al., 1995). *In-vitro* studies in rats revealed that E enhances CFTR expression (Rochwerger et al., 1994). In contrast to E, E plus P decreases CFTR mRNA expression in glandular epithelia of guinea pigs (Mularoni et al., 1995). Mularoni et al., (1995) reported that P suppresses CFTR expression *in vivo*. The effects of P on CFTR expression could also be due to down-regulation of CFTR activities (Chan et al., 2001).

Cyclic changes in CFTR expression correlates with cyclic changes in uterine fluid volume (Chan et al., 2009). Enhanced CFTR expression resulted in a maximum volume of uterine fluid at proestrus and oestrus stages of oestrous cycle (Rochwerger & Buchwald, 1993). On the other hand, suppression of CFTR expression related to the minimum uterine fluid volume as observed at metoestrus and dioestrus stages (Rochwerger & Buchwald, 1993). CFTR communicates with epithelial sodium channel (ENaC) in out-of-phase fashion. The out-of-phase expression of CFTR and ENaC in the uterus may be of physiological importance. Down-regulation of CFTR and up-regulation of ENaC may explain for the fluid loss observed at dioestrus (Krzeminski et al., 1998). CFTR has also been described as a negative regulator of ENaC since low expression of CFTR at dioestrus may further increase ENaC function to facilitate uterine fluid reabsorption (Chan et al., 2001).
Figure 2.12: Role of CFTR in regulating fluid volume and HCO₃⁻ secretion in mouse uterus with functional implications in various reproductive events such as sperm transport, capacitation, embryo development and blastocyst implantation. Water flow following the electrolyte gradient into the lumen facilitates sperm transport while HCO₃⁻ promote capacitation of sperm in the lumen. Fluid reabsorption may take place when epithelial sodium channel (ENaC) is up-regulated such as during implantation. Increasing HCO₃⁻ concentrations, provides an optimal microenvironment for sperm capacitation, fertilization and embryo development to take place in the oviduct.

Source: (Chan et al., 2009)

2.4.2.2 Aquaporin

Water can be transported across the epithelial cell by transcellular or paracellular pathways. The paracellular pathway is through tight junctions (TJs) (Claude & Goodenough, 1973) while transcellular pathway involves specialized membrane transporters known as aquaporins (Aqp). Figure 2.13 showed that Aqp is a specialized
transporter that has greater capacity to transport water across the epithelial membrane by transcellular pathway compared to simple diffusion (Agre et al., 2002).

Figure 2.13: Aquaporins water channel. When compared with simple diffusion across the lipid bilayer of the plasma membrane (A, left panel), Aqp water channels (A, right panel) provide much more rapid water transport across the plasma membrane. Aqp offers various possibilities for the regulation of water transport: by trafficking of Aqp between the plasma membrane and a pool of intracellular vesicles (B, left panel), or by changing permeability of Aqp residing in the plasma membrane (B, right panel).

Source: (Zelenina et al., 2005)

Aqp is a hydrophobic, intrinsic membrane protein with a low molecular weight between 26 to 34 kDa that facilitates rapid and passive movement of H₂O (Denker et al., 1988). To date, thirteen Aqp isoforms were reported in male (Wilson et al., 2013) and female (He et al., 2006) reproductive tissues in rats, mice, marmosets and humans. On the basis of permeability, Aqp has been divided into three groups: (a) aquaporins-selective water permeable consist of Aqp-0, Aqp-1, Aqp-2, Aqp-4, Aqp-5, Aqp-6, and Aqp-8 (b) aquaglyceroporins-water and glycerol permeable consist of Aqp-3, Aqp-7, Aqp-9, and Aqp-10 and (c) superaquaporins- consist of Aqp-11 and Aqp-12 (Ishikawa
et al., 2006). In the uterus, sex steroids could influence the expression of several Aqp isoforms, including Aqp-1, 5 and 7. Their specific pattern of expression suggests their role in water movement between intraluminal, interstitial and capillary compartments (Jablonski et al., 2003; Branes et al., 2005; Lindsay & Murphy, 2006).

Aqp-1 is a 28-kDa protein and most commonly expressed in red blood cells, kidneys, lungs, brain and uterus (Agre et al., 1987; Denker et al., 1988). In rat uterus, Aqp-1 was localized to an inner circular layer of the smooth muscle, endothelial cells and mostly concentrated in mesometrial part of myometrium during implantation (Lindsay & Murphy, 2004a). This subunit is up-regulated by E and participates in H₂O reabsorption and secretion across the secretory epithelia (Li et al., 1997). A study involving both layers of myometrium showed that a slight increase in Aqp-1 expression was observed in E treated mice while a slight decrease in this protein expression was reported in the P-treated rats (Jablonski et al., 2003). In control and P-treated animals, Aqp-1 was localized to the inner circle layer of myometrium.

Aqp-5 is a member of classic aquaporin isoform that was reported to be expressed in ovaries, oviducts and uterus (Skowronska, 2010). In pigs, uterine Aqp-5 expression was influenced by P (Skowronska et al., 2009). In rats, uterine Aqp-5 was redistributed at the apical plasma membrane of the uterus at the time of implantation under the influence of P (Lindsay & Murphy, 2004b). Additionally, Aqp-5 was found to be redistributed to the apical membrane on day 6 and 7 of pregnancy (Lindsay & Murphy, 2004b). In ovariectomised rats, expression of Aqp-5 protein increases at the apical membrane of uterine epithelial cells under P influence (Lindsay & Murphy, 2006). The combined treatment of E and P in ovariectomised rats showed a similar increase in the pattern of Aqp-5 expression consistent with the reported increase in its
mRNA expression (Richard et al., 2003). Besides that, there is evidence showing the involvement of Aqp-5 in uterine fluid reabsorption under P influence.

Aqp-7 is a non-selective H$_2$O channels that regulate the transport of H$_2$O, glycerol, urea and other small non-electrolytes. This Aqp isoform is also expressed in uteri, granulosa cells and oocytes (Zhang et al., 2012). Uterine Aqp-7 participates in decidualisation while ovarian Aqp-7 participates in H$_2$O movement into the antral follicle, that assists the antrum formation (Huang et al., 2006).

In the uterus, H$_2$O transport through Aqp is important for regulating the amount of fluid volume within the uterine lumen (Lindsay & Murphy, 2004a). The movement of luminal fluid into the endometrial stroma occurs through apical membrane Aqp (Enders & Schlafke, 1967).
CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

3.1.1 Animal

All procedures involving experimental animals were approved by the University of Malaya Institutional Animal Care and Use Committee (IACUC), with ethics number: FIS/27/01/2012/MHM (R). Three month-old adult female Sprague-Dawley (SD) rats and Wistar Kyoto (WKY) rats, weighing 225 ± 25 g were obtained from the Animal House, Faculty of Medicine, UM. The rats were caged under standard conditions (lights at 06:00 to 18:00 h; room temperature 24°C; 4 animals per cage). The animals were fed with rat chow diet (Harlan, Germany) and tap water ad libitum.

3.1.2 Chemicals and consumable

The chemicals and consumables used were analytical graded (AR) and were shown in Table 3.1
<table>
<thead>
<tr>
<th>No</th>
<th>Chemicals</th>
<th>Company</th>
</tr>
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<tr>
<td>1</td>
<td>Testosterone propionate (Testosterone)</td>
<td>Sigma, St. Louis, MO</td>
</tr>
<tr>
<td>2</td>
<td>Progesterone (100 mg, purity minimum 99 %)</td>
<td>Sigma, St. Louis, MO</td>
</tr>
<tr>
<td>3</td>
<td>17β-oestradiol (oestrogen)</td>
<td>Sigma, St. Louis, MO</td>
</tr>
<tr>
<td>4</td>
<td>Peanut oil (P2144, 1L)</td>
<td>Sigma, St. Louis, MO</td>
</tr>
<tr>
<td>5</td>
<td>Flutamide purity≥ 98%</td>
<td>Sigma, St. Louis, MO</td>
</tr>
<tr>
<td>6</td>
<td>Finasteride purity ≥ 98%</td>
<td>Sigma, St. Louis, MO</td>
</tr>
<tr>
<td>7</td>
<td>Tetramethylenediamine (TEMED)</td>
<td>Sigma, St. Louis, MO</td>
</tr>
<tr>
<td>8</td>
<td>Pro-Prep Protein Extraction Solution</td>
<td>Intron Biotechnology, Korea</td>
</tr>
<tr>
<td>9</td>
<td>Micro BCA Protein Assay Kit</td>
<td>Thermo Scientific, Rockford, USA</td>
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<tr>
<td>10</td>
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<td>Bio Rad, Alfred Nobel Drive Hercules, CA, USA</td>
</tr>
<tr>
<td>11</td>
<td>Poly Vinylidene Fluoride (PVDF) membrane</td>
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<tr>
<td>12</td>
<td>Bovine Serum Albumin (BSA)</td>
<td>Innovative, Peary Court Novi, Michigan, USA</td>
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<td>13</td>
<td>Spectra Multi-Colour Broad Range</td>
<td>Fermentas, USA</td>
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<td>RNA later</td>
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<tr>
<td>15</td>
<td>RNase Free Water</td>
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<tr>
<td>16</td>
<td>Micro 96 well plate and cover</td>
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<td>17</td>
<td>High capacity RNA to cDNA</td>
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<td>26</td>
<td>Cyclic AMP EIA Kit</td>
<td>Cayman Chemical Company, Ann Arbor</td>
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</table>
3.1.3 Sterilization

All experiment consumables (plastic tips, collection tubes, glass disposables) were sterilised by autoclaving at 1.05 kg/cm² (15 psi) on liquid cycle for 20 minutes. Distilled water (dH₂O) and ultrapure water (Mili-Q or ddH₂O) were used for critical parts of the study.

3.2 Animal surgical procedures

3.2.1 Anaesthesia

For ovariectomy procedure, the rats were anaesthetized by using ketamine (80 mg/kg) and xylazine (8 mg/kg). Ketamine is a hydrochloric salt, which is used for general anaesthesia in combination with a sedative such as xylazine. Xylazine is a drug that used for relaxation, pain-killing and general anaesthesia in animals. The combination of these drugs was injected intraperitoneally (IP). After injection of ketamine and xylazine, the rats were placed in left lateral position. The depth of anaesthesia was monitored by the loss of plantar reflexes while breathing movement was maintained.

3.2.2 Ovariectomy

Ovariectomy was performed to eliminate endogenous steroid hormones. The ovaries were located at the caudal end of the ribs on lateral sides of the animal. The rat was placed on a heat pad to maintain its core body temperature, while the rectal temperature was continuously observed. The area for ovariectomy was shaved, then swabbed and cleaned with 70% ethanol to remove the filth. The location of the ovarian fat pad was confirmed before making the incision through the overlying muscle layers. The tip of
double sharp iridectomy scissors was inserted through the muscle layer and the muscle fibers were separated by opening the scissors in a dorsal-ventral direction. The edge of the incision was held and opened with a small tooth forceps. Then, ovary was pulled out through the incision with a blunt forceps by grasping the surrounding fat pads. The junction between the oviduct and the uterus was ligated. The ovary was removed by using a scissor. The muscle layer was sutured and the skin incision was closed with wound clips. The animal was turned over and the procedure was repeated on the other side.

To prevent any post-surgical wound infection, the animals were given 0.1 ml Kombitrim 240 antibiotic via intramuscular injection (Parhizkar et al., 2008). The animals were then kept individually per cage with free access to food and water ad libitum. All drugs treatment was started at least 21 days following ovariectomy to eliminate the effect of endogenous sex-steroids as previously described (Salleh et al., 2005).

3.3 Identification of oestrus cycle

Vaginal secretions were collected by using a plastic pipette filled with 10 μl of normal saline (NaCl 0.9%). The tips of the pipette was inserted into the rat vagina, but not deep to avoid cervical stimulation. The unstained material was placed onto a slide and was observed under a light microscope. The proportion of different cell types was used to determine oestrus cycle phases, where round and nucleated cells are epithelial cells, which define proestrus; irregular shape cells without nuclei are cornified cells that were observed during oestrus; little round cells are leukocytes that characterize dioestrus. At metoestrus however, three different kinds of cells were observed (Figure 3.1).
Figure 3.1: Identification oestrous cycle phases in rat. (A) proestrus (B) oestrus (C) metoestrus (D) dioestrus under 40-fold magnification.

Source: (García P et al., 2011)
3.4 Animals and hormone treatment

This study was divided into three main parts: (A) investigating the effects of testosterone on uterine fluid volume and ionic compositions, (B) investigating the effects of testosterone on endometrial receptivity in ovariectomised rats receiving sex steroid replacement mimicking hormonal changes in early pregnancy, (C) investigating the effects of testosterone on endometrial receptivity and embryo implantation in intact early pregnant rats. Figure 3.2 shows a flow chart of the experimental design.

![Flow chart of the experimental design in this study.](image)

**Figure 3.2:** Flow chart of the experimental design in this study.

T has been reported to exert multiple effects on female reproductive organs. It plays an important role in regulating normal physiological process in female such as decidualisation and maintaining follicular health (Cardenas et al., 2002). However, high levels of T could interfere with the peri-implantation embryo and uterine developments resulting in early pregnancy loss. In view of these conditions, supraphysiological levels
of T were studied to investigate the adverse effects of T on the uterine fluid regulation and endometrial receptivity that may lead to infertility.

In fact, testosterone propionate was used in this study. Subcutaneous administration of testosterone was made rather than the silastic implants as in our study, testosterone was acutely administered to the rats (maximum of five days) which did not require sustained release via silastic implant.

Administration of 1mg/kg/day testosterone was regarded as a supraphysiological dose in female rats according to Dehghan et al. (2014). Meanwhile, administration of 1 µg/kg/day 17β-oestradiol and 16 mg/kg/day progesterone were intended to achieve a physiological plasma level of both hormones in female rats (Salleh et al., 2005). All drugs were dissolved in 0.1 ml peanut oil prior to subcutaneous administration behind the neck scruff.

Flutamide and finasteride, which are the androgen receptor (AR) blocker and 5α-reductase inhibitor, respectively were administered together with T. Flutamide is a non-steroidal potent androgen receptor blocker that displays pure antiandrogenic activity, without exerting agonist or some other hormonal activity (Singh et al., 2000). Meanwhile, administration of finasteride inhibited the conversion of T to DHT, thus reflecting the involvement of DHT in mediating T effect on the uterus. (Glina et al., 2004). Additionally, DHT was not commercially available (prohibitable substance).
3.4.1 Investigating the effects of testosterone on uterine fluid volume and ionic compositions

Rats were divided into the following groups (n=6 per group):

Group 1: 3 or 5 days peanut oil, vehicle (C)

Group 2: 3 or 5 days 1 mg/kg/day testosterone (T)

Group 3: 3 or 5 days 1 µg/kg/day 17β-oestradiol (E)

Group 4: 3 days combined 1 µg/kg/day 17β-oestradiol plus 1 mg/kg/day testosterone (E+T)

Group 5: 3 days 1 µg/kg/day 17β-oestradiol followed by 2 days 1 mg/day testosterone E+T

Group 6: 3 days 1 µg/kg/day 17β-oestradiol followed by 2 days 1 mg/kg/day testosterone plus 2.5 mg/kg/ day finasteride (5α-reductase inhibitor) E+ (T+FIN)

Group 7: 3 days 1 µg/kg/day 17β-oestradiol followed by 2 days 1 mg/kg/day testosterone plus 5mg/kg/ day flutamide (androgen receptor (AR) blocker) E+ (T+FLU)

Group 8: 3 days 1 µg/kg/day 17β-oestradiol followed by 2 days peanut oil (E+V)

Group 2 and 3 were designed to investigate effects of individual sex-steroids. Group 1 acted as a control for group 2 and 3. Group 4 was designed to investigate the combine effects of T with E. Group 5 was designed to investigate the effects of T on uteri exposed to E, which caused fluid to accumulate within the uterine lumen (Chinigarzadeh et al., 2014). Group 6 was designed to investigate dihydrotestosterone (DHT) involvement in mediating T effect and group 7 was designed to investigate androgen receptor (AR) involvement in mediating T effect. Group 8 acted as a control for group 5-7.
3.4.2 Investigating the effects of testosterone on endometrial receptivity in ovariectomised rats receiving sex steroid replacement mimicking hormonal changes in early pregnancy

In this study, ovariectomised adult female Wistar-Kyoto (WKY) rats were used which received sex steroid replacement regime that mimics the hormonal profile in early pregnancy. To mimic the hormonal profile in early pregnancy, E and P were administered according to the established protocol by Kennedy (1986) however with slight modifications.

In brief, the basic protocol involved injection of 1.0 µg/kg/day E on day 1 and day 2, 1.0 µg/kg/day E and 4 mg/kg/day P on day 3, no treatment on the 4th and 5th day, 16 mg/kg/day P and 0.5 µg/kg/day E on the next 3 days (days 6–8). Vehicle treated animals (control) received daily injections of peanut oil. 1 mg/kg/day T, was administered for three (3) days from day 6-8 which was considered as the days of uterine receptivity with and without flutamide (5 mg/kg/day) or finasteride (1 mg/kg/day) where both inhibitors were administered 30 minutes prior to the T injection. (n=6 per group)

![Figure 3.3: Protocol of sex-steroid replacement regime. Schematic representations of time-course of steroid treatment protocol administered to ovariectomised rats to mimic the fluctuating steroid hormone levels in early pregnancy. (n=6 per group)](image-url)
3.4.3 Investigating the effects of testosterone on endometrial receptivity and embryo implantation in intact early pregnant rats

In this study, female rats that showed at least two consecutive, regular four-day oestrus cycles were used. Proestrus adult female Sprague-Dawley (SD) rats, weighing 225 ± 25 g were caged overnight with a male of the same species and proven fertile. In the morning of the following day, successful copulation was confirmed by the presence of a vaginal plug that contained spermatozoa, and the day was designated as day 1 of pregnancy.

Rats were randomly assigned to these groups (n=12 per group):

i. Pregnant rats with no treatment, (C)
ii. Pregnant rats treated with 250 µg/kg/day of T, (T250)
iii. Pregnant rats treated with 500 µg/kg/day of T, (T500)

Group 2 and 3 were treated for 3 consecutive days starting from day 1 to day 3 of pregnancy, which represent the early pregnancy period. The rats from all groups were sacrificed on day 4 and day 6 of pregnancy (n=6 for each day). The uterine horns were excised on day 4 and the uteri were analysed for morphological changes, expression, and distribution of uterine receptivity markers. Day 6 uteri were examined to determine the number of embryo implantation sites.
Table 3.2: Summary of animal and hormonal treatments

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<tr>
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<tr>
<td>1</td>
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<td>3 or 5 days peanut oil (C)</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>3 or 5 days 1 mg/kg/day T (T)</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>3 or 5 days 1 µg/kg/day E (E)</td>
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<tr>
<td>4</td>
<td></td>
<td>3 days combined 1 µg/kg/day E + 1 mg/kg/day T (E+T)</td>
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<tr>
<td>5</td>
<td></td>
<td>3 days 1µg/kg/day E followed by 2 days 1 mg/day T E+T</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>3 days 1 µg/kg/day E followed by 2 days 1 mg/kg/day T + 2.5 mg/kg/ day FIN E+ (T+FIN)</td>
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<tr>
<td>7</td>
<td></td>
<td>3 days 1 µg/kg/day E followed by 2 days 1 mg/kg/day T plus 5mg/kg/ day FLU E+ (T+FLU)</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>3 days 1 µg/kg/day E followed by 2 days peanut oil (E+V)</td>
</tr>
<tr>
<td>Intact pregnant</td>
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<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
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<td>2</td>
<td></td>
<td>Pregnant rats treated with 250 µg /kg/day of T, (T250)</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>Pregnant rats treated with 500 µg /kg/day of T, (T500)</td>
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</table>
3.5  *In-vivo uterine perfusion*

To investigate changes in the rate of fluid and Cl\(^-\) secretions in uterus, in-vivo perfusion of the uterine horn was performed according to the previously described method by Salleh et al. (2005). In brief, anaesthetized animals were placed on a heat pad to maintain their body temperature constant and an incision was made at flanks to insert an in-going tube pre-filled with perfusate at the uterotubal junction which is the distal end of the uterine horns. An out-going tube was inserted and tied in-situ at the uterocervical junction. A syringe-driven infusion pump (Harvard Apparatus, USA) was used to deliver perfusate into the lumen at a constant rate of 0.75 µl/min. The in-going tube, uterine horn and out-going tube were placed at the same level to minimize the gravitational effect. The perfused fluid was collected over a period of 3 hours into a small, pre-weighed 1.5 ml Eppendorf® Safe-Lock microcentrifuge tubes (Figure 3.4).

The content of perfusate was as follows: 110.0 mmol/L NaCl, 14.3 mmol/L Na\(_2\)HCO\(_3\), 1.0 mmol/L Na\(_2\)HPO\(_4\), 15 mmol/L KCl, 0.8 mmol/L MgSO\(_4\), 10.0 mmol/L HEPES, 1.8 mmol/L CaCl\(_2\), 5.5 mmol/L glucose, pH 7.34. These compositions were selected to closely mimic the normal uterine fluid composition.

In order to confirm the involvement of CFTR in mediating fluid and Cl\(^-\) secretion, CFTRinh-172 a specific inhibitor for CFTR (Sigma–Aldrich, St. Louis, USA) was added to the perfusate at 10 mM concentration. CFTRinh-172 is a potent and selective blocker of the CFTR and it is 500-fold more potent as compared to other CFTR blocker, glibenclamide (Taddei et al., 2004).
Figure 3.4: Picture showed the procedures of uterine perfusion. (A) In-going tube was inserted into the uterine lumen at the uterotubal junction. (B) Uterine fluid was collected into the eppendorf tube for 3 hours.

3.5.1 Analysis of uterine fluid

To determine changes in the fluid secretion rate, the net weight of collecting fluid was divided by total perfusion period (180 minutes). The tube was weighed with an electronic balance (EL3002 METTLER TOLEDO) prior to and after perfusate collection. The rate of fluid secretion was calculated from the difference between tube weight prior to and after perfusate collection over a period of 3 hours.

3.5.2 Analysis of uterine fluid concentration changes by F-Dextran

Fluorescein-dextran (F-dextran; MW 450 kDa) was dissolved (1.0 mmol/L) in the perfusion medium to act as a non-absorbable marker to detect changes in fluid concentration (Naftalin et al., 2002). The sample was diluted 1:250 in PBS and mixed well in a miniature cuvette. The cuvette was then placed in a Hitachi (F-2000) spectrophotometer which was set at a wavelength of Ex=488 nm, EM=520 nm. The
procedure was repeated triplicate for each sample. Spectrophotometer calibration was carried out by a serial dilution of the perfusate.

3.5.3 Analysis of chloride concentration

Cl⁻ concentration changes in the perfusate was directly measured by using ion selective electrodes (ISE, Hanna Instruments, Singapore) according to the method by Gholami et al., 2013.

3.6 Measurement of uterine cAMP levels

Freshly isolated uterine tissues were homogenized in five volumes (ml/g tissue) of ice-cold trichloroacetic acid (TCA) using Polytron-type homogenizer. Homogenate was centrifuged at 3000 rpm for 10 minutes, and the supernatant was transferred into test tubes and extracted three times by using water-saturated ether. The ether fraction was discarded and the residual ether in aqueous fraction was removed by heating at 70°C for 5 minutes. cAMP in aqueous fractions was measured by using an enzyme-linked immunosorbent assay (ELISA) method (Cayman Chemical Company, Ann Arbor; Item No. 581001) according to manufacturer's guidelines. Absorbance was read at 405 nm in a microplate reader (iMark; Bio - Rad, Hercules, CA, USA) and the unit was expressed in pmol/ml. Cayman’s cAMP assay is a competitive ELISA that permits cAMP measurement with a range from 0.3-750 pmol/ml and sensitivity of approximately 3 pmol/ml. The antibody also has 100% cross-reactivity with the corresponding cAMP (Cyclic AMP ELISA Kit Protocol, Cayman).
3.7 Measurement of blood serum hormone levels

Blood samples were collected into a separator tube (SST) and were allowed to clot for 30 minutes at room temperature. The samples were centrifuged at 5000 rpm, for 15 minutes. Serum samples were aliquoted and stored at -20°C. Serum samples were analysed in triplicate by using Rat Progesterone, Rat Testosterone and Rat Estrogen enzyme-linked immunosorbent assay (ELISA) kits (CUSABIO - USA). ELISA was performed according to manufacturer's guidelines. Absorbance was determined by using a microplate reader (iMark; Bio - Rad, Hercules, CA, USA) at a wavelength of 450 nm. A set of standard serial dilutions of known concentrations was provided by the manufacturer and were used to construct a standard curve to determine the hormone levels. Standard curves were created by serial dilution and hormone concentrations in the samples were determined from the standard curve. For the sensitivity, the minimum detectable dose of rat T is typically less than 0.06 ng/ml, rat E less than 15.6 pg/ml and rat P is less than 0.2 ng/ml (ELISA Kit, CUSABIO-USA Protocol). Meanwhile, for LH and FSH levels, the serum was sent to Clinical Diagnostic Laboratory of University Malaya Medical Centre for the hormone levels measurement. Sex-steroid hormone levels were not measured prior to ovariectomy due to fluctuation in endogenous estrogen and progesterone levels, while after ovariectomy, the serum levels of 17β-oestradiol and progesterone were 69.7±0.12 pg/ml and 23.67±0.13 ng/ml respectively (Dehghan et al., 2014).
3.8 Immunohistochemistry and histological procedures

3.8.1 Paraffin block preparation

A day after the last day of hormone treatment, rats were humanely sacrificed by cervical dislocation. The right uterine horn was immediately harvested and fixed in 4% paraformaldehyde (PFD) at 4°C for 4–5 hours. A standardized region of the horn (mid-portion) was processed using an automated tissue processing machine (Leica, Germany). Table 3.3 shows the steps involved in tissue processing.

Table 3.3: Steps for tissue processing

<table>
<thead>
<tr>
<th>Step</th>
<th>Solution</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50% Ethanol</td>
<td>1 hour</td>
</tr>
<tr>
<td>2</td>
<td>70% Ethanol</td>
<td>1 hour</td>
</tr>
<tr>
<td>3</td>
<td>80% Ethanol</td>
<td>1 hour</td>
</tr>
<tr>
<td>4</td>
<td>95% Ethanol</td>
<td>1 hour</td>
</tr>
<tr>
<td>5</td>
<td>95% Ethanol</td>
<td>1 hour</td>
</tr>
<tr>
<td>6</td>
<td>Absolute Ethanol</td>
<td>1 hour</td>
</tr>
<tr>
<td>7</td>
<td>Absolute Ethanol</td>
<td>1 hour</td>
</tr>
<tr>
<td>8</td>
<td>Xylene I</td>
<td>1 hour</td>
</tr>
<tr>
<td>9</td>
<td>Xylene II</td>
<td>1 hour</td>
</tr>
<tr>
<td>10</td>
<td>Xylene III</td>
<td>1 hour</td>
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<tr>
<td>11</td>
<td>Wax I</td>
<td>1 hour</td>
</tr>
<tr>
<td>12</td>
<td>Wax II</td>
<td>1 hour</td>
</tr>
</tbody>
</table>
After processing, tissues were embedded in paraffin wax (Surgipath Paraplast, Leica, melting point 56°C). All these processes were performed using an embedding machine (Histo-Center LL-N, Thermolyne). Then, the paraffin wax was left to cold in the freezer, forming a block. When the wax is frozen and formed a block, the mould was removed. All blocks were put in a refrigerator before sectioning.

The blocks were trimmed before sectioning which was done by using a microtome (ARM 3600, Histo-Line Laboratories) and the range of sectioning was between 8-10 µm. In the sectioning process, the sections were cut and the ribbons produced were placed in a water bath (35-37°C). Then, using slides, the floating ribbon section were fished out. The slide was then labelled with a proper identification according to its group. Before haematoxylin and eosin (H&E), immunohistochemistry (IHC) or immunofluorescence (IF) stainings, the slides were dried in an oven. The tissues sectioned for IHC and IF were placed on polylysine-coated slides (Menzel, Germany).

### 3.8.2 Hematoxylin and Eosin staining

5 µm in thick sections were prepared and mounted onto glass slides. The sections were stained with hematoxylin and eosin (H&E). Prior to H&E staining, the slides were deparaffinised in three changes of xylene and rehydrated in the reducing concentration of ethanol (100%, 95%, 80%, and 70%) for 3 minutes each. The slides were washed with distilled water and then stained with Mayer’s hematoxylin solution for 20 minutes. The sections were rinsed in running tap water until no excessive hematoxylin and stained with eosin for 5 minutes. Finally, the sections were dehydrated in graded alcohol (70%, 80%, 95% and 100%), followed by two changes of xylene for 3 minutes each and mounted with DPX solution. H&E staining is a common staining technique in histopathology that uses a combination of the two dyes. H&E can demonstrate the
nucleus and cytoplasmic inclusions in specimens as Hematoxylin stained the nucleus appeared light blue while Eosin imparted a pink colour to the cytoplasm.

The slides were then visualized under a light microscope. Outer and inner uterine circumferences were measured by using a NIS-Elements AR program (Nikon Instruments Inc., Melville, NY, USA). To compare between circumferences of the uterine lumen of rats with different weight and sizes, ratio of inner to outer circumferences was determined. All images were captured by Nikon Eclipse 80i (Japan) camera attached to a light microscope.

### 3.8.3 Immunohistochemistry

Immunohistochemistry (IHC) is a widely used technique to visualize the distribution of proteins in tissues.

#### 3.8.3.1 Deparaffinisation and tissue hydration

The tissues were cut into 5 µm sections and applied to slides. Prior to immunodetection, the slides were immersed in the xylene bath for 5 minutes to dissolve the paraffin from the tissue section. The sections were then hydrated in decreasing concentrations of ethanol (90%, 70 % & 50%) for 10 min each. The slides were washed with PBS for 5 minutes to ensure complete removal of alcohol and the excess liquid was aspirated from slides.
3.8.3.2 Blocking of endogenous peroxidase

Tri-sodium citrate (pH 6.0) was used for antigen retrieval. The samples were heated with tri-sodium citrate at 95º C for 10 minutes. The slides were allowed to cool in the buffer for approximately 20 minutes. Prior to using horseradish peroxidase as a labelled antibody, it is important to block any endogenous peroxidase present in the tissue sections. The endogenous peroxidase was blocked with 1% H₂O₂ in PBS for 30 minutes. The slide was then thoroughly washed in PBS for 5 minutes.

3.8.3.3 Incubation with blocking serum, primary antibodies and secondary antibodies

Non-specific binding of the sections was blocked with 1.5% blocking serum of the animal in which the secondary antibody is raised. Blocking step helps to minimize non-specific binding of the secondary antibody within the cell. In addition, it is important to ensure that these blocking proteins do not originate from the species in which the primary antibody was raised. Otherwise, specificity of the secondary antibody to the primary antibody will be lost. The slides were then incubated with primary antibody in 1.5% BSA overnight at 4°C in a humidified chamber. The following day, the sections were gently rinsed and immersed in PBS buffer three times, 5 minutes each. The sections were incubated with biotinylated secondary antibody for 1 hour at room temperature and then exposed to avidin and biotinylated HRP complex (Santa Cruz, California, USA) in PBS for another 30 minutes.
3.8.3.4 Visualization and counterstaining

The sections were incubated in DAB (Diaminobenzidine HCl) (Santa Cruz, California, USA) for 10 minutes, which gave dark-brown stains, or until the desired stain intensity was reached. Individual slides were monitored to determine the appropriate development time. To achieve an end-point counterstaining, the sections were washed in deionised H₂O for 5 minutes. The slides were then counter-stained in Mayer’s formulation hematoxylin for 5–10 seconds, immediately wash with several changes of deionised H₂O. Finally, the sections were dehydrated in graded ethanol (70%, 80%, 95% and 100%, followed by xylene twice for 1 minute each and mounted with DPX solution. The slides were left overnight in the fume hood to dry.

Immunofluorescence is an antigen-antibody reaction in which the antibodies are tagged with a fluorescent dye and antigen-antibody complex is visualized by using fluorescence microscope. Fluorochromes are dyes that absorb rays and emit visible light that known as fluorescence. Commonly fluorochromes use is include fluorescein (FITC) which was excited by a blue (wavelength 488nm) light and emitted a green (520nm) colour signals.
3.9 Western blotting

Western blotting is a widely accepted technique used to measure the amount of specific proteins in the samples. Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate different proteins in a given sample. The separated proteins are then blotted onto a matrix where they are stained with antibodies that target a specific protein. By analysing the location and intensity of the specific reaction, expression details of the target proteins in tissue homogenate could be obtained. The western blotting steps are described in below.

3.9.1 Protein extraction

Total protein was extracted using the PRO-PREP (Intron, Korea) solution kit. This kit is able to isolate protein from whole tissue. The whole uterine tissues were snapped frozen in liquid nitrogen and then stored at -80°C prior to protein extraction. 30 mg of uterine tissue was weighed and soaked in 400μl PRO-PREP (Intron, Korea) extraction solution in the presence of protease inhibitors. The tissue was homogenized by using a homogenizer for 5 minutes and then incubated at -20°C for 20 minutes to induce cell lysis and increase the protein concentration. Total cell protein was obtained by centrifugation at 13000 rpm for 15 minutes at 4°C that was used to separate the supernatant which was then stored at -20°C.

3.9.2 Protein quantification

Protein quantification was performed to determine the concentration of sample by using a Micro Bicinchoninic Acid (BCA) Protein Assay kit (Thermo Scientific™-US). The kit contains Micro BCA Reagent A (MA), Micro BCA Reagent B (MB), Micro
BCA Reagent C (MC) and Bovine Serum Albumin (BSA) as standard ampules at 2.0 mg/ml in a solution of 0.9% saline and 0.05% sodium azide. The detergent-compatible bicinchoninic acid formulation was used for colorimetric detection and quantitation of total protein. A purple-colored reaction was formed by chelation of two molecules of BCA with one cuprous ion (Cu⁺). This water-soluble complex exhibits a strong absorbance at 570 nm wavelength that is linear with increasing protein concentrations. The sample concentrations were calculated from a linear plot of BCA standard concentration of the intensity (X-axis-) against changes in colour in absorbance. This absorbance was obtained from the reaction between BSA and BCA reagents. Standard concentrations of BSA were prepared in 10, 8, 6, 4, 2, 1, 0.5, and 0 µg/ml. The samples then were diluted 1:50 with PBS buffer. 50µl of total protein samples or standard as a serial dilution were added to 50 µl of BCA reagents in a Greiner UV Transparent 96 Well Plate (Thermo Scientific™ - US) in triplicate. The plate was incubated for 2 hours at 37°C, and then the intensity of colors was quantified by using a plate reader at a wavelength of 570 nm. A standard curve was generated by serial dilution and sample protein concentration was calculated from the standard curve.

3.9.3 Western blot analyses

One-dimensional electrophoresis was used to separate most proteins and nucleic acids. Location of protein in electrophoresis was based on charges, mass, size and strength of the electrical field (Bolt & Mahoney, 1997). The matrix in polyacrylamide gel is a polymer of acrylamide and bis- acrylamide (Sigma-US). The presence of N, N, N’, N’ - tetramethylethylenediamine (TEMED) and ammonium per sulphate (APS) catalyses gel polymerization. Ionic detergent Sodium Dodecyl Sulfate (SDS) was used in polyacrylamide gel. SDS denatures the protein and makes them negatively charged.
The charge is equally distributed on the protein molecules and aid protein migration according to their sizes.

3.9.3.1 SDS gel preparation

SDS gel consists of two different parts, which are the resolving, and the stacking parts. The resolving part was placed at the bottom of gel caster with a pH of 8.8 while the stacking part was placed above the resolving part with a pH of 6.8. The stacking gel was used to pack protein together after loading and mostly prepared in 4%. 10% and 12% resolving gels were used in this study. The compositions of each gel were shown in Table 3.4.

<table>
<thead>
<tr>
<th>Stock solution</th>
<th>Resolving Gel 10% (μl)</th>
<th>Resolving Gel12% (μl)</th>
<th>Stacking Gel 4% (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH20</td>
<td>3800</td>
<td>3200</td>
<td>2975</td>
</tr>
<tr>
<td>Acrylamide 30%</td>
<td>3400</td>
<td>4150</td>
<td>670</td>
</tr>
<tr>
<td>1.5M Tris (pH 8.8)</td>
<td>2600</td>
<td>2600</td>
<td>1250</td>
</tr>
<tr>
<td>10% SDS</td>
<td>100</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>10% APS</td>
<td>100</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>TEMED</td>
<td>10</td>
<td>10</td>
<td>5</td>
</tr>
</tbody>
</table>

3.9.3.2 Sample preparation and electrophoresis

30 μg proteins for each sample was mixed with loading dye and boiled for 5 minutes. Gel-cast was placed inside the electrophoresis tank and bathed in migration buffer with the recommended time as instructed by the manufacturer. Pre-stained protein marker was loaded into one well to assess protein band size. Following that, the boiled samples were loaded onto the gel. Electrophoresis was started and allowed to run at 100V for 120 minutes. Electrophoresis was then stopped when the front dye reached the bottom of the gel.
3.9.3.3 Protein transfer

Following electrophoresis, the proteins were transferred onto Polyvinylidene fluoride (PVDF) membrane (BIORAD, USA) for immunoblotting. The hydrophobicity of PVDF makes it an ideal support for proteins in electrophoresis and dot blotting applications. The membrane was soaked in methanol for 2 minutes and then into towbin buffer for another 2 minutes. The towbin buffer contains 20% methanol, which functions to promote dissociation of SDS from the protein and improve absorption of protein onto the membrane (Pettegrew et al., 2009). The gel and membrane were sandwiched between sponges and filter papers and were clamped tightly to ensure no air bubbles formed between the gel and the membrane. The sandwich was submerged in transfer buffer to which an electrical field was applied at a constant voltage of 100 V for 120 minutes. The negatively charged proteins travel towards the positively charged electrode with the membrane stopping the movements.

3.9.3.4 Blocking the membrane

Following protein transfer, the membrane was exposed and washed with double distilled water (ddH₂O) for 5 minutes. Then, the membrane was blocked with BSA for 90 minutes at room temperature. Blocking prevents non-specific background binding of primary or secondary antibodies. Then, the membrane was rinsed with phosphate buffered saline with Tween-20 (PBST) for 5 minutes.
3.9.3.5 Incubation with primary and secondary antibodies

The primary antibody was diluted in ratio 1:1000 in PBST buffer and incubated with the membrane overnight at 4°C with agitation. The next day, the membrane was washed three times in PBST while agitating, 5 minutes or more per wash to remove residual primary antibody. After washing, the membrane was incubated with HRP-conjugated secondary antibody diluted in 1:2000 ratios in PBST for 60 minutes under agitation. Once again, the membrane was washed three times in PBST while agitating, 5 minutes or more per wash to remove secondary antibody residue.

3.9.3.6 Visualization and analysis of the protein bands

Opti - 4CN™ Substrate kit was used to visualize the target protein band. This kit contains colorimetric (HRP) substrate and enables visualization of the bands with naked eyes. The visualized blots were captured by a gel documentation system (Vilber Lourmat, from Fisher Scientific, USA) and density of each band was determined using the Image J software NIH (version 1.46j; National Institutes of Health, Bethesda, MD, USA). The ratio of each target band/reference was calculated and was considered as the expression levels of the target proteins. Primary and secondary antibodies used in this study were shown in Table 3.5.
Table 3.5: Primary and secondary antibodies used in Western blot (WB), Immunohistochemistry (IHC) and Immunofluorescence (IF).

<table>
<thead>
<tr>
<th>Target protein</th>
<th>Primary antibody</th>
<th>Secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IHC and IF : 1:100 dilution</td>
<td></td>
</tr>
<tr>
<td>CFTR</td>
<td>Goat polyclonal CFTR (SC-8909)</td>
<td>rabbit anti-goat IgG-HRP (SC-2922)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rabbit anti-goat IgG-FITC (SC-2777)</td>
</tr>
<tr>
<td>MECA-79</td>
<td>Rat monoclonal MECA-79 (SC-19602)</td>
<td>rabbit anti-rat HRP (SC-2303)</td>
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<td></td>
<td></td>
<td>rabbit anti-rat IgG-B (SC-2041)</td>
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<tr>
<td>Claudin-4</td>
<td>Goat polyclonal claudin-4 (SC-17664)</td>
<td>rabbit anti-goat IgG-HRP (SC-2922)</td>
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<td></td>
<td></td>
<td>rabbit anti-goat IgG-FITC (SC-2777)</td>
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<tr>
<td>Occludin</td>
<td>Goat polyclonal occludin (SC-8145)</td>
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<td></td>
<td>rabbit anti-goat IgG-FITC (SC-2777)</td>
</tr>
<tr>
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<td>Goat polyclonal mucin-1 (SC-6827)</td>
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<td>rabbit anti-goat IgG-FITC (SC-2777)</td>
</tr>
<tr>
<td>αvβ3 integrin</td>
<td>Rabbit polyclonal αvβ3 integrin (Cat. No. 251672)</td>
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<td></td>
<td></td>
<td>Goat anti-rabbit IgG-FITC (SC-2012)</td>
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<td>E-cadherin</td>
<td>Goat polyclonal E-cadherin (SC-8426)</td>
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<tr>
<td>Msx-1</td>
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<td>rabbit anti-goat IgG-HRP (SC-2922)</td>
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<td>β-actin</td>
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<tr>
<td>GAPDH</td>
<td>Rabbit polyclonal GAPDH (SC-25778)</td>
<td>goat anti-rabbit IgG-HRP (SC-2030)</td>
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</table>
3.10 Real time quantitative PCR

Real-time quantitative PCR (RT-qPCR) allows sensitive, specific and reproducible quantitation of nucleic acids. In this method, RNA was first transcribed into complementary DNA (cDNA) by reverse transcriptase from total RNA. The cDNA was then used as the template for qPCR reaction. RT-qPCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production at each PCR cycle (in real time) as opposed to endpoint detection.

3.10.1 Sample collection

At the end of the treatment, uterine horns were removed and washed with PBS buffer. Following that, tissues were soaked in five volumes of RNA Later® (Ambion, L/N: 1206029) solution. RNA later® solution was used to stabilize and protect tissues cellular RNA. Samples can be preserved in RNA Later® solution at -20°C or below up to 2 years. Total RNA was extracted from 30 mg tissues (wet weight) in the RNA later® solution using the RNeasy Plus Mini Kit (cat. no. 74134) (QIAGEN, Germany).

3.10.2 RNA extraction

RNeasy Plus Mini Kit (cat. no. 74134) (QIAGEN, Germany) is a kit for phenol-free total RNA extraction from tissues where genomic DNA contamination is removed. The kit consists of Buffer RLT Plus, Buffer RW1, Buffer RPE, RNase-Free Water, gDNA Eliminator Mini Spin Columns, RNeasy Mini Spin Columns, Collection Tubes (1.5 ml) and (2 ml). Firstly, tissues were retrieved from RNA later® solution with sterile forceps and drip-dried immediately with an absorbent paper towel. About 30 mg wet tissues were weighted, lysed and homogenized by using homogenizer in highly denaturing
guanidine-isothiocyanate-containing Buffer RLT Plus, which immediately inactivated RNases to ensure isolation of intact RNA. The lysate was centrifuged for 3 minutes at 13000 rpm. Carefully, the supernatant was removed by pipetting and homogenized sample was transferred to a gDNA eliminator spin column that was placed in a 2 ml collection tube. The lysate was centrifuged for 30 seconds at 10000 rpm. Then, the column was discarded and the flow-through was kept. 600 μl of 70% ethanol was added to the flow-through and mixed well by pipetting. 70% ethanol was added to provide appropriate binding conditions for RNA. 700 μl of the sample including the precipitate was transferred onto a RNeasy spin column placed in a 2 ml collection tube. The lid was closed and centrifuged for 15 seconds at 10000 rpm. The flow-through discarded and 700 μl of Buffer RW1 was added to the RNeasy Mini spin column in a 2 ml collection tube. Again, the lid was closed and centrifuged for 15 seconds at 10000 rpm. Two times of 500 μl of Buffer RPE were added to the RNeasy spin column for two times and centrifuged for 15 seconds followed by 2 minutes at 10000 rpm. The flow-through was discarded and the sample was centrifuged at full speed for 1 minute to further drying the membrane. The RNeasy spin column was placed in a new 1.5 ml collection tube and 30–50 μl RNase-free water was added directly to the spin column. The lid was closed and centrifuged for 1 minute at 10000 rpm to elute the RNA. The samples were kept in -20°C until further use.

### 3.10.3 RNA quality control

DNA, protein or phenol that could inhibit the RNA downstream solicitation, might contaminate the isolated RNA. Therefore, evaluation of RNA integrity and quality is important. The purity and concentration of RNA were evaluated by using A260/280 UV absorption ratios (Thermo Scientific NanoDrop™ spectrophotometer). The
microvolume capability of NanoDrop™ spectrophotometers allowed quick and easy run quality control checks on nucleic acid and protein samples. In addition, the instrument’s short measurement cycle and generally easier to use greatly increases the rate at which samples can be processed, making it possible to implement multiple quality control checks throughout a procedure or processes. The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of DNA and RNA. A ratio of ~2.0 was generally accepted as “pure” for RNA. If the ratio was appreciably lower, it could indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm.

In addition, an agarose gel is another method to evaluate the RNA integrity. Each RNA sample was assessed by determining the integrity of 1% ethidium-bromide agarose gel and evaluated by electrophoresis in TBE buffer. After running the agarose gel for 45 minutes at 90 V, the gel was visualized in a specific gel documentation system (Vilber Lourmat, Fisher Scientific). RNA sharp and clear bands can be seen as 18S and 28S rRNA. DNA ladder was used to identify the location of the RNA band at 1 Kb.

### 3.10.4 Conversion of RNA to cDNA

RNA samples were converted to cDNA by using the High capacity RNA to cDNA kits (Applied Biosystems, US). This kit consists of a 2X reverse transcription (RT) buffer mix and 20X RT enzyme mix. Total RNA was converted to cDNA by adding up to 9μl of RNA (1000 ng), 10μl of RT buffer, 1μl of RT enzyme and nuclease free-water to make up to 20μl reaction mixtures. The reactions were mixed and placed on ice. The tube was centrifuged to spin down the contents and to eliminate air bubbles. The thermal cycler was used to perform conversion. The reaction was loaded into a thermal
cycler that was programmed at 37°C for 60 minutes, 95 °C for 5 minutes and hold at 4°C (Kwok & Higuchi, 1989). Following completion, total cDNA was stored in -20°C for long-term storage.

### 3.10.5 Running the Real-time Quantitative PCR

Two-step Real-time PCR was run to evaluate mRNA expression according to manufacturer procedures. The amplified region of cDNA was tagged with the fluorescence-labelled probe. The specificity of primer and probe ensures that the expression of target DNA was specifically evaluated. TaqMan probe has a sensitivity of 100% and a specificity of 96.67% (Tsai et al., 2012) and is capable of detecting as few as 50 copies of RNA/ml and as low as 5-10 molecules (Hofmann-Lehmann et al., 2002). All reagents including probes and primers were obtained from Applied Biosystems, USA. The primers were designed by the same company and were listed in Table 3.6.

To carry out the reaction, 1µl of cDNA, 5µl of master mix buffer, 0.5µl of target primer, and 3.5µl of RNase-free water were mixed and loaded into 96 well plates. The total volume of PCR mixture in each well was 10µl and all experiment samples were loaded in triplicates. After that, the reaction plate was loaded into StepOnePlus™ Real-Time PCR Systems. Real time-PCR program was set according to the manufacturer’s protocol which included 2 minutes at 50°C reverse transcriptase, 20 seconds at 95°C activation of polymerase, denaturation at 95°C for 1 second and annealing at 60°C for 20 seconds. Denaturing and annealing steps were performed for 40 cycles.
3.10.6 Data analyses for quantification of gene expression

The data was analyzed according to comparative fold changes as previously described by Wong and Medrano (2005):

\[ \Delta \text{Ct} = \text{Ct of target gene} - \text{Ct of housekeeping genes average} \]

\[ \Delta \Delta \text{Ct} = \Delta \text{Ct of treated samples} - \Delta \text{Ct of non-treated samples} \]

Fold changes = Ct \( 2^{\Delta \Delta \text{Ct}} \)

Amplifications of target and reference were measured in samples and reference. Measurements were standardized by using GenEx software. The relative quantity of target in each sample was determined by comparing the normalised target quantity in each sample to normalise target quantity in references. Data Assist v3 software from Applied Biosystems was used to calculate RNA folds changes.

<table>
<thead>
<tr>
<th>Assay ID</th>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>mRNA size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rn01455968_m1</td>
<td>Cftr</td>
<td>Cystic fibrosis transmembrane conductance regulator (CFTR)</td>
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<td>Aqp1</td>
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<td>Aquaporin-5</td>
<td>1426</td>
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<tr>
<td>Rn00569727_m1</td>
<td>Aqp7</td>
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<td>Rn00596601_m1</td>
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<td>Muc1</td>
<td>Mucin-1</td>
<td>2328</td>
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<td>Occludin</td>
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<td>Hprt1</td>
<td>Hypoxanthine phosphoribosyltransferase-1 (HPRT1)</td>
<td>1260</td>
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</tbody>
</table>
3.11  **Electron microscopy**

The electron microscope uses a beam of electrons to create an image of the specimen. It is capable of much higher magnification and has a greater resolving power compared to the light microscope, allowing it to visualize much smaller objects with fine detail. Transmission electron microscopy (TEM) is widely used in cell biology where the electron beam passes through the sample. The beam will then be absorbed and scattered to produce a contrast and an image.

### 3.11.1 Field emission scanning electron microscopy (FESEM)

Field emission scanning electron microscope (FESEM) is widely used to visualize very small topographic details on the surface or entire objects. This technique was used to observe structures that may be as small as 1 nm. FESEM was performed to evaluate the presence of microstructures on the surface. For FESEM preparation, uteri were cut longitudinally into 2 mm × 2 mm section and fixed at least 24 hours in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) at 4°C. The specimens were air-dried and then placed on carbon planchets and examined with a FEG Quanta 450 EDX-OXFORD, Field Emission Scanning Electron Microscope, Netherlands at 5 kV, under low vacuum and at working distances of 11.0 mm and at magnifications of 3000×. Photographs were taken in slow scanning mode at 1280 × 1024 pixels.
3.11.2 Transmission electron microscopy (TEM)

3.11.2.1 Primary fixation, rinsing and secondary fixation of specimen

Uteri were cut into 2 mm cross sections and prefixed by immersing the specimens in a 2.5% glutaraldehyde solution prepared in 0.1 M cacodylic acid buffer (pH 7.4) and incubated at 4°C overnight. In order to remove excess glutaraldehyde from the samples, specimens can be washed in 0.1 M cacodylic acid buffer (pH 7.4), starting with one time for 10 minutes, and then three times for 15 minutes at 4°C. The process of fixation helps to preserve the structure of specimen with no alteration from living state and it is crucial for biological specimens. The specimens were stabilized for transmission electron microscopy (TEM) investigation by post-fixation with 1% osmium tetroxide prepared in 0.1 M cacodylic acid buffer (pH 7.4) for 1.5 hours at room temperature.

3.11.2.2 Dehydration the specimen

The specimens were dehydrated in 50% ethanol for 10 minutes, 70% ethanol for 10 minutes, 80% ethanol for 10 minutes, 90% ethanol for 10 minutes, and absolute ethanol twice for 15 minutes at room temperature. This process allowed water in the samples to be slowly exchanged through liquids with lower surface tensions.

3.11.2.3 Infiltration with transitional solvent, resin and embedding

Samples were incubated twice for 5 minutes in propylene oxide and then transferred to a rotor for 1 hour at room temperature in a 1:1 mixture of propylene oxide and epon [47% Embed 812, 31% DDSA (dodeny succinic anhydride), 19% NMA (nadic methyl anhydride), 3% BDMA (benzyldimethylamine)]. Then, the samples were incubated overnight in 1:2 propylene oxide-epon. The next day, the samples were incubated in
100% epon for 2 hours. Individual uterine samples were embedded in 100% epon in silicon flat embedding molds, and capsules were polymerized in 60°C oven for 48 hours.

3.11.2.4 Sectioning, staining and viewing of specimen

Semi-thin sections were cut into 0.5 μm to 2 μm and were stained with toluidine blue for 1 minute on a hot plate (70°C to 90°C). The sections were examined by light microscope and used for identifying the specimen within the resin block before proceeding with ultramicrotomy. Ultrathin transverse sections (70 nm) were prepared using a diamond knife (Diatome) on a MT 6000-XL ultramicrotome, captured on 300-mesh copper grids, and stained with 2% uranyl acetate. All reagents and materials were obtained from Electron Microscopy Sciences (Hatfield, PA, USA). The ultrathin sections were observed under a transmission electron microscope (Libra 120 Zeiss, Oberkochen, Germany).

3.12 Determination of implantation sites

The sites of implantation at day 6 were identified by using Chicago Sky Blue Dye (Sigma-Aldrich, Dorset, UK). The dye was injected intravenously in 0.85% sodium chloride to the rats under anaesthesia, 1 ml of dye was perfused through the tail vein. Chicago Sky Blue stained areas of high vascular permeability such as implantation sites. Images of sites (blue stained spots in uterus) and inter-implantation sites (unstained spaces between two blue-stained bands) were then captured.
3.13 Statistical analysis

For statistical analyses, one-way analysis of variance (ANOVA) with Tukey’s post hoc test was used. Probability levels of less than 0.05 (p < 0.05) were considered as significant. Post-hoc statistical power analysis was performed and all values were >0.8 which indicated adequate sample size (n=6).
CHAPTER 4: EFFECTS OF TESTOSTERONE ON UTERINE FLUID VOLUME AND IONIC COMPOSITIONS

4.1 Background

Female sex hormones are known to regulate the fluid and ionic compositions of uterine fluid. 17β-oestradiol (E) increases (Clemetson et al., 1977) while progesterone (P) decreases the fluid volume in the uterus (Salleh et al., 2005). In humans, the amount of fluid in the uterus was higher at around the time of ovulation under the influence of E (Cloke et al., 2008). The increased in fluid and ionic concentration mainly Cl⁻ and HCO₃⁻ are essential for processes of reproduction such as sperm transport, capacitation, fertilization, embryo transport and implantation (Chan et al., 2002). Under P influence, reduced amount of fluid could assist embryo attachment during implantation (Naftalin et al., 2002). The regulation of fluid and ionic composition mainly Cl⁻ and HCO₃⁻ in uterus involves ion channels including CFTR (Cl⁻ and HCO₃⁻ transport) and Aqp (H₂O transport).

CFTR, a cAMP-dependent Cl⁻ channel was reported to mediate fluid and Cl⁻ secretions in the uterus (Chan et al., 2002; Gholami et al., 2012). CFTR gene mutation results in Cystic Fibrosis (CF), an autosomal recessive disorder characterized by defective Cl⁻ and fluid secretions (Alvarez et al., 2013). CFTR was expressed at the apical membrane of endometrial luminal epithelia (Salleh et al., 2005). In addition, CFTR also mediates uterine HCO₃⁻ secretion. Uterine CFTR expression is under the control of sex-steroids, down-regulated by P and up-regulated by E (Gholami et al., 2012). CFTR expression was also found to fluctuate at different stages of the oestrus cycle, highest at proestrus and oestrus and lowest at dioestrus (Chan et al., 2002; Gholami et al., 2012).
Aquaporin (Aqp) is a small hydrophobic, intrinsic membrane protein with the low molecular weight of between 26 to 34 kDa, facilitates rapid and passive movement of H$_2$O (Denker et al., 1988). In the uterus, expression of several Aqp isoforms, including Aqp-1, 5 and 7 has been reported to be influenced by sex hormones (Jablonski et al., 2003). Aqp-1 is the most commonly expressed isoform (Denker et al., 1988). Aqp-1 was found to be distributed in the uterine stroma vasculature and was upregulated by E (Li et al., 1997). Aqp-5, a classic aquaporin isoform was reported to be expressed in ovaries, oviducts and uterus (Skowronski, 2010). In pigs, uterine Aqp-5 expression was shown to be influenced by P (Skowronski et al., 2009) while in rats, redistribution of Aqp-5 was observed in the uterus under the P influence (Lindsay & Murphy, 2006). Aqp-7, a non-selective H$_2$O channels regulates the transport of H$_2$O, glycerol, urea and other small non-electrolytes, was also expressed in the uterus (Zhang et al., 2012). Aqp-7 participates in decidualisation (Huang et al., 2006).

In this study, I hypothesized that high testosterone (T) level in females interfere with normal fluid and Cl$^-$ secretions in the uterus. I further hypothesized that T inhibits activity and expression of CFTR, which could interfere with normal fluid and Cl$^-$ secretions. Additionally, T could also affect uterine Aqp-1, 5 and 7 expressions.

Therefore, in this part, I aimed to investigate the effect of T on uterine fluid and Cl$^-$ secretion, CFTR and Aqp-1, 5 and 7 expressions. Additionally, I also aimed to investigate T effect on the uterine cAMP level that can affect CFTR functional activity. These findings were important as they could help to explain the mechanisms underlying testosterone effect on embryo implantation.
4.2 Results

4.2.1 Uterine fluid and Cl⁻ secretion rate

Figure 4.1: Changes in the fluid secretion rate as measured by (a) gravidometric method and (b) F-dextran concentration changes. The highest fluid secretion was observed in rats treated with E. Treatment with T resulted in a significant decrease in the rate of fluid secretion and an increase in F-dextran concentration changes which was antagonized by flutamide but not finasteride. C = control, T = testosterone, E = 17β-oestradiol, FLU = flutamide, FIN = finasteride, V = peanut oil. *p < 0.05 as compared to E, #p < 0.05 as compared to E + T, †p < 0.05 as compared to without CFTRinh-172. n=6 per treatment group. Data presented as mean ± SEM.
CFTRinh-172 is a specific inhibitor for CFTR, which was used to confirm the involvement of this protein in mediating fluid and Cl− secretion in the uterus. Meanwhile, F-dextran is a non-absorbable marker that detects any changes in fluid volume. Changes in F-dextran reflect either an increase or decrease in fluid volume that suggests that either secretion or absorption take place in uterus. Figure 4.1 (a) and (b) shows the rate of uterine fluid secretion as directly measured (µl/min) or through changes in the concentration of F-dextran marker. Based on Salleh et al, (2005), the two parameters well correlated between one another. The changes were the highest in E-treated rats. Administration of CFTRinh-172 significantly reduced the rate of fluid secretion and the decrease in F-dextran concentration in E-treated animals (p < 0.05). In rats receiving 3 days E followed by 2 days T-only treatment, E+T, a significant reduction in the rate of fluid secretion and an increase in F-dextran concentration were observed as compared to 3 days E followed by 2 days peanut oil treatment, E+V, which was not inhibited by CFTRinh-172. Concomitant administration of T with flutamide, E+(T+FLU) but not finasteride, E+(T+FIN) resulted in a significant increase in fluid secretion rate and decrease in F-dextran concentration as compared to T-only treatment which was antagonized following administration of CFTRinh-172. The fluid secretion rate was slightly lesser and F-dextran concentration was slightly higher in E+V treatment as compared to the group, which received E-only treatment and was also inhibited by CFTRinh-172.

In Figure 4.2, Cl− concentration was the highest in E-only treatment group followed by the E+V treatment. In both groups, the increase in Cl− concentration was inhibited by CFTRinh-172. Administration of E+T resulted in a significant reduction in Cl− concentration, which was not inhibited by CFTRinh-172. Concomitant administration of E+(T+FLU) resulted in higher Cl− concentration as compared to T-only treatment group and was inhibited by CFTRinh-172. E+(T+FIN) administration, however did not
result in a significant change in Cl− concentration as compared to T-only treatment group with no significant effect was observed following administration of CFTRinh-172.

Figure 4.2: Changes in uterine fluid. Cl− content. The highest Cl− concentration was seen in E treated group, which was significantly reduced following administration of CFTRinh-172. E+V treatment resulted in a slight decrease in Cl− concentration as compared to E-only treatment, which was decreased following CFTRinh-172 application. T caused a decrease in Cl-concentration; however, this effect was not antagonized by CFTRinh-172. Administration of CFTRinh-172 decreases the Cl− concentration in the group which received concomitant T and flutamide treatment. C = control, T = testosterone, E = 17β-oestradiol, FLU = flutamide, FIN = finasteride, V = peanut oil. *p < 0.05 as compared to E, #p< 0.05 as compared to E + T, †p < 0.05 as compared to without CFTRinh-172. n = 6 per treatment group. Data presented as mean ± SEM.

4.2.2 cAMP level

cAMP is a known stimulator of CFTR functional activity. In Figure 4.3, the level of cAMP was the highest in E-treated group. Concomitant treatment of (E+T) resulted in a significant decrease in cAMP level. Similarly, treatment with E+T also resulted in a decrease in the cAMP level. Treatment with E+V also resulted in a significant decrease
in cAMP level as compared to E treatment. Meanwhile, treatment with E+(T+FLU) but not E+(T+FIN) resulted in a significant increase in cAMP level as compared to the group receiving T treatment. The lowest cAMP level was noted in the T-only treatment group.

Figure 4.3: cAMP level in uterine tissue homogenates. The highest cAMP level could be seen in the group which received E-only treatment. Administration of T resulted in a significant decrease in cAMP level. Flutamide administration resulted in an increase in cAMP level in uterine tissue homogenates in rats receiving T treatment. C = control, T = testosterone, E = 17β-oestradiol, FLU = flutamide, FIN = finasteride, V = peanut oil. *p < 0.05 as compared to E, #p < 0.05 as compared to E + T. n = 6 per treatment group. Data presented as mean ± SEM.

4.2.3 Uterine morphological changes

The mid portion of the uterine horns showed an obvious increase in the size of the uterine luminal following E. E+V treatment resulted in significant fluid accumulation within the uterus. Meanwhile, the luminal of the rat uteri in groups receiving E+T or E+(T+FIN) treatment were almost obliterated, indicating a significant reduction in the fluid amount in the uterus. A significantly smaller luminal circumference was seen in the group receiving E+T as compared to E+V. Meanwhile, treatment with E+(T+FLU)
resulted in a significantly larger lumen as compared to T-only treatment (Figure 4.4). In Table 4.1, the ratio of inner luminal/outer circumference was the highest in the group receiving E followed by E+V treatment. The lowest ratio was observed in the group receiving T-only treatment. E+T treatment resulted in 1.37 times lower ratio as compared to E+V treatment. E+(T+FLU) administration resulted in a significant increase in the inner luminal/outer circumference ratio as compared to E+T treatment. E+(T+FIN) however, had no significant effect was noted on the ratio.

**Figure 4.4:** Histological appearance of uterine lumen in different group of treatment. A significantly larger lumen with multiple folds could be seen in E as compared to E+V treatment groups, with the latter having less folded inner lining than the former. A significantly smaller lumen could be seen in the E+T treated rats as compared to E-treatment alone. The size of uterine lumen was significantly larger in the group receiving T and flutamide treatment as compared to T-only treatment. C = control, T = testosterone, E = 17β-oestradiol, FLU = flutamide, FIN = finasteride, V = peanut oil. Scale bar = 250 µm. n = 6 per treatment group
Table 4.1: The relative size of the uterine lumen in rats receiving different steroid treatment. C = control, T = testosterone, E = 17β-oestradiol, FLU = flutamide, FIN = finasteride, V = peanut oil. *p < 0.05 as compared to E, **p < 0.05 as compared to E + T. n = 6 per treatment group. Data presented as mean ± SEM.

<table>
<thead>
<tr>
<th>Group</th>
<th>Ratio of luminal/outer uterine circumference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>0.359±0.045*</td>
</tr>
<tr>
<td>T</td>
<td>0.459±0.008*</td>
</tr>
<tr>
<td>E</td>
<td>0.898±0.069</td>
</tr>
<tr>
<td>(E+T)</td>
<td>0.653±0.025*</td>
</tr>
<tr>
<td>E+T</td>
<td>0.517±0.035*</td>
</tr>
<tr>
<td>E+(T+FIN)</td>
<td>0.482±0.057*</td>
</tr>
<tr>
<td>E+(T+FLU)</td>
<td>0.579±0.012**</td>
</tr>
<tr>
<td>E+V</td>
<td>0.797±0.013*</td>
</tr>
</tbody>
</table>

4.2.4 CFTR distribution and expression

4.2.4.1 CFTR protein distribution

Figure 4.5 (a) and (b) shows CFTR distribution in the uterus at the epithelia lining of the uterine lumen. The highest intensity of dark-brown/fluorescence staining was observed in E-only treatment group followed by the group receiving E+V treatment. Mild staining intensity was seen in the group receiving E+T treatment. Concomitant administration of E+(T+FLU) resulted in a relatively higher staining intensity as compared to E+T treatment. Meanwhile, no significant difference in intensity was observed in the group receiving E+(T+FIN) treatment as compared to T-only treatment. Lower intensity was observed in the group receiving concomitant treatment with (E+T) and T-only treatment.
Figure 4.5: CFTR distribution as observed by (a) immunoperoxidase and (b) immunofluorescence in the uterus in different treatment group. A higher staining intensity could be seen at the apical membrane in rats receiving E treatment as compared to E+V and E plus T plus flutamide treatment. A low staining intensity could be seen in the groups receiving T-only treatment as compared to E treatment. L = lumen, C = control, T = testosterone, E = 17β-oestradiol, FLU = flutamide, FIN = finasteride, V = peanut oil (vehicle). NC = negative control. Scale bar = 50 µm. n=6 per treatment group.
4.2.4.2 CFTR protein expression

**Figure 4.6:** Uterine CFTR protein expression in rats receiving different sex-steroid treatment. The highest expression was seen following E-only treatment followed by E+V treatment. CFTR protein expression was reduced following administration of T to E-treated rats, however, were increased following concomitant flutamide and T administration. Low CFTR expression was seen in the control and T-only treatment group. Molecular weight for CFTR = 165 kDa, β-actin = 43 kDa. C = control, T = testosterone, E = 17β-oestradiol, FLU = flutamide, FIN = finasteride, V = peanut oil. *p < 0.05 as compared to E, #p < 0.05 as compared to E + T. n = 6 per treatment group. Data presented as mean ± SEM.

The expression level of CFTR protein in uterine tissue homogenates was the highest following E only treatment followed by E+V treatment (Figure 4.6). CFTR expression was the lowest in the group which received T only treatment and E+T group. Concomitant administration of E+(T+FLU) resulted in higher CFTR expression as compared to E followed by T-only treatment. No differences in band density were observed between group receiving E+(T+FIN) as compared to E+T treatment.
4.2.4.3 CFTR mRNA expression

Figure 4.7: Uterine CFTR mRNA expression in rats receiving different steroid treatment. The highest CFTR mRNA expression was observed following E-only treatment followed by E+V treatment. The expression was reduced following administration of T in rats pre-treated with E, however, this was antagonized by flutamide administration. C = control, T = testosterone, E = 17β-oestradiol, FLU = flutamide, FIN = finasteride, V = peanut oil. *p < 0.05 as compared to E, #p < 0.05 as compared to E + T. n = 6 per treatment group. Data presented as mean ± SEM.

Figure 4.7 shows changes in CFTR mRNA expression, which was the highest in E-only treatment group followed by E+V treatment. Very low expression was noted in the group receiving T-only treatment. E+T treatment resulted in a significant decrease in CFTR mRNA expression as compared to E-only treatment. Meanwhile, in the group receiving E+(T+FLU), a significant increase in CFTR mRNA expression was observed however no significant increase was noted following administration of E+(T+FIN).
4.2.5 Aquaporin 1, 5 and 7 distribution and expression

4.2.5.1 Aqp-1 protein distribution

**Figure 4.8**: Distribution of Aqp-1 protein in the uterus as observed by immunofluorescence. Stromal, myometrial and epithelial distribution could be seen under E influence. Under T influence, fluorescence signal could be seen mainly in epithelia lining the uterine lumen. Higher signal could be seen in E+T treated rats as compared to E+V treatment. Arrows pointing toward Aqp-1 protein distribution. L = lumen. C = control, T = testosterone, E = 17β-oestradiol, FLU = flutamide, FIN = finasteride, V = peanut oil (vehicle). NC = negative control. Scale bar = 50 µm. n = 6 per treatment group.
Figure 4.8 shows the Aqp-1 distribution in the uterus. High distribution was seen in epithelia lining the uterine lumen of rats receiving E-only and T-only treatment. In E-only treated rats, high signals could also be seen in stroma and myometrium. However, in rats receiving T-only treatment, faint signals in the stroma could be seen. In rats receiving (E+T) treatment, moderate signals could be seen in epithelial lining the uterine lumen with no signal seen in the stroma. In rats receiving E+T treatment, moderate signals could be seen in epithelial lining the uterine lumen and stroma. Administration of 17β-oestradiol followed by combined testosterone and finasteride E+(T+FIN) to the rats resulted in lower stromal and epithelial signals as compared to the rats receiving E+T treatment. In rats receiving 17β-oestradiol followed by combined testosterone and flutamide treatment E+(T+FLU), very low signals were seen in both stroma and luminal epithelia.
4.2.5.2 Aqp-1 mRNA expression

**Figure 4.9:** Levels of expression of Aqp-1 mRNA in uterine tissue homogenates. The highest mRNA expression levels were noted in rats receiving E-treatment. Aqp-1 mRNA levels in rats receiving E+T were higher than E+V. C = control, T = testosterone, E = 17β-oestradiol, FLU = flutamide, FIN = finasteride, V = peanut oil (vehicle). *p<0.05 as compared to control, †p<0.05 as compared to E, #p<0.05 as compared to E+V, φ p<0.05 as compared to E+T. n = 6 per treatment group. Data presented as mean ± SEM.

Figure 4.9 shows Aqp-1 mRNA expression levels in uterine tissue homogenates of rats receiving different treatment. The level of Aqp-1 mRNA was 1.95 and 1.75 fold higher following E-only and T-only treatment respectively as compared to control. Concomitant (E+T) treatment resulted in lower levels of Aqp-1 mRNA expression as compared to E or T-only treatment. In rats receiving E+T treatment, the levels of expression of Aqp-1 mRNA were three folds higher than the rats receiving E+V treatment. In these rats, treatment with E+(T+FLU) resulted in approximately two folds lower Aqp-1 mRNA expression levels as compared to E+T treatment. A slight decrease in levels of Aqp-1 mRNA expression were noted in rats receiving E+(T+FIN) treatment as compared to E+T treatment.
4.2.5.3 Aqp-5 protein distribution

Figure 4.10: Distribution of Aqp-5 protein in the uterus as observed by immunofluorescence. Under T influence, high Aqp-5 distribution was observed at apical and basolateral membrane of uterine luminal epithelia. Under E influence, very low distribution of Aqp-5 could be seen. L = lumen. C = control, T = testosterone, E = 17β-oestradiol, FLU = flutamide, FIN = finasteride, V = peanut oil (vehicle). NC = negative control. Scale bar = 50 μm. n=6 per treatment group.
Figure 4.10 shows Aqp-5 distribution in the uterus. High distribution could be seen at apical and basolateral membranes of luminal epithelia and stroma in T-only and E+T treated rats. Additionally, the myometrial distribution could also be seen. In rats receiving E+(T+FIN) treatment, high stromal distribution and the moderate epithelial distribution could be seen. In rats receiving E+(T+FLU) treatment, moderate distribution could be seen in stroma and low distribution in luminal epithelial. In E and E+V treated rats, very low signal could be seen in the luminal epithelia. Moderate distribution could be seen in the luminal epithelia following concomitant (ET) treatment as compared to E-only treatment.

4.2.5.4 Aqp-5 mRNA expression

![Figure 4.11](image_url)

**Figure 4.11:** Levels of expression of Aqp-5 mRNA in uterine tissue homogenates. The highest expression level was noted in T-only treated rats. E+T treatment resulted in higher Aqp-5 mRNA expression level as compared to E+V treatment. C = control, T = testosterone, E = 17β-oestradiol, FLU = flutamide, FIN = finasteride, V = peanut oil (vehicle). *p<0.05 as compared to control, †p<0.05 as compared to E, #p<0.05 as compared to E+V, φ p<0.05 as compared to E+T. n = 6 per treatment group. Data presented as mean ± SEM.
Figure 4.11 shows changes in Aqp-5 mRNA expression levels in uterine tissue homogenates of rats receiving different treatment. Aqp-5 mRNA expression was the highest in rats receiving T-only treatment which was approximately three folds higher as compared to control. In rats receiving E-only treatment, Aqp-5 mRNA levels were not significantly different as compared to control. Concomitant (E+T) treatment resulted in mRNA expression levels to be markedly reduced as compared to T-only treatment. The levels were however not significantly different as compared to E-only treatment. In rats receiving E+T treatment, levels of Aqp-5 mRNA were higher than the rats receiving E+V treatment (p<0.05). In rats receiving E+(T+FIN) treatment, no significant changes in Aqp-5 mRNA levels were observed as compared to the rats receiving E+T treatment. However, marked reduced Aqp-5 mRNA levels were noted in rats receiving E+(T+FLU) as compared to E+T treatment.
4.2.5.5 Aqp-7 protein distribution

**Figure 4.12:** Distribution of Aqp-7 protein in the uterus as observed by immunofluorescence. In T-treated group, high Aqp-7 distribution was observed in luminal epithelia and stroma. Low Aqp-7 distribution could be seen following E-only treatment. L = lumen. C = control, T = testosterone, E = 17β-oestradiol, FLU = flutamide, FIN = finasteride, V = peanut oil (vehicle). NC = negative control. Scale bar = 50 µm. n=6 per treatment group.
Figure 4.12 shows the distribution of Aqp-7 in the uterus. Aqp-7 was highly distributed in luminal epithelia and stroma in rats receiving T-only treatment. In rats receiving E-only treatment, very low epithelial distribution could be seen. Low distribution was also seen in luminal epithelia of rats receiving combined (E+T) treatment. In E+T treated rats, mild to moderate distribution could be seen in the epithelial lining the uterine lumen. In rats receiving E+ (T+FLU) treatment, very low epithelial distribution was seen while in rats receiving E+ (T+FIN) treatment, lower epithelial distribution was also seen relative to E+T treatment.

4.2.5.6 Aqp-7 mRNA expression

![Aqp-7 mRNA expression diagram]

**Figure 4.13**: Levels of expression of Aqp-7 mRNA in uterine tissue homogenates. The highest mRNA levels were noted in T-only treated rats. Aqp-7 mRNA expression levels in E+T treated rats were higher than E+V treated rats. C = control, T = testosterone, E = 17β-oestradiol, FLU = flutamide, FIN = finasteride, V = peanut oil (vehicle). *p<0.05 as compared to control, †p<0.05 as compared to E, #p<0.05 as compared to E+V, φp<0.05 as compared to E+T. n = 6 per treatment group. Data presented as mean ± SEM.

Figure 4.13 shows changes in Aqp-7 mRNA expression level in the uterus of rats receiving different treatment. Aqp-7 mRNA level was the highest in rats receiving T-only treatment (nearly four-folds higher as compared to control). In rats receiving E-
only treatment, Aqp-7 mRNA levels were not significantly different from control. Concomitant (E+T) administration resulted in markedly reduced Aqp-7 mRNA expression as compared to T-only treatment. In rats receiving E+T treatment, levels of Aqp-7 mRNA were higher as compared to E+V treatment (p<0.05). In E+(T+FIN) treated rats, Aqp-7 mRNA levels were not significantly different from the rats receiving E+T treatment. However, in rats receiving E+(T+FLU) treatment, levels of Aqp-7 mRNA were significantly lower as compared to E+T treatment.
4.3 Discussion

The findings from this study indicated that T administration to ovariectomised, sex-steroid-replaced rats could interfere with uterine fluid volume and concentration of Cl⁻. T could exert the inhibitory effect on E-induced fluid and Cl⁻ secretions in the rat uteri. T resulted in a significant decrease in fluid and Cl⁻ secretions via inhibiting the E-induced CFTR expression and functional activities. These evidences include reduced amount of fluid within the uterine lumen, lack of inhibitory effect of CFTRinh-172, down-regulation of CFTR protein and mRNA expression, reduced level of cAMP and reduced CFTR distribution at the apical membrane of luminal epithelia in rats receiving T-treatment. There was marked reduction in fluid secretion in the uterus under T influence as indicated by both gravidometric and colorimetric measurements of uterine fluid volume. Under condition of T influence, administration of CFTRinh-172, a specific inhibitor for CFTR in the perfusate produced no significant inhibition on fluid and Cl⁻ secretion, which indicate that CFTR functional activity was absent at the luminal surface of endometrium. CFTRinh-172 was reported to inhibit CFTR channel opening by interacting with arginine 347, adjacent to the cytosolic surface of CFTR (Caci et al., 2008). CFTRinh-172 was proven effective in preventing toxin-induced fluid and Cl⁻ secretion in the small intestines (Thiagarajah et al., 2004). I have shown for the first time that the functional role of CFTR in uterus in-vivo was reduced by testosterone by using a specific CFTR inhibitor, CFTRinh-172. This inhibitor was much more specific than the previous CFTR inhibitor glibenclamide, which was also shown to inhibit fluid and Cl⁻ secretion in the uterus under the E influence (Gholami et al., 2013).

I have also found that the CFTR was distributed at the apical membrane of luminal epithelial, which support its role in mediating fluid and Cl⁻ secretion under the E influence. CFTR function requires cAMP (Chan et al., 1999). I have found the levels of cAMP were elevated under E influence and were significantly inhibited by T. cAMP
binds protein kinase, which then phosphorylates CFTR (King et al., 2012). T may inhibit cAMP production resulting in reduced CFTR activity. In this study, I have shown that the effect of T was mediated via AR binding due to the inhibitory effects of flutamide, which is a potent androgen blocker on T action. However, the effect of T did not involve the T active metabolites 5α-DHT. The inability of T to mediate its action via 5α-DHT could be due to the absence of 5α-reductase activity in the uterus (Giannopoulos, 1973). A similar effect was reported in female pelvic floor muscle, which was responsive only toward T and not 5α-DHT (Max et al., 1981). Based on these observations, T could therefore be the major androgens affecting the function of the female reproductive system and not 5α-DHT, which is the main androgen affecting the male reproductive functions. In humans, uterine AR expression, known to be up-regulated by E (Pelletier et al., 2004) was highest during the secretory phase of the menstrual cycle (Bai et al., 2008). T preferentially binds to AR rather than being aromatised to E. In-vitro study of endometrial epithelial cells further shows that E binds to cytoplasmic AR prior to translocation into the nucleus (Giannopoulos, 1973). Although the physiological level of T is required for uterine events such as decidualisation, the supraphysiological level could produce adverse effect on uterine function such as inhibition of uterine fluid and Cl⁻ secretions and CFTR expression. A significantly higher inhibition of T as compared to peanut oil as observed in this study confirmed that this effect was due to T rather than the declining level of E.

This study has shown that expression of Aqp-1, 5 and 7 mRNA and proteins in the uterus were markedly increased under T influence (compared to control). However, under the influence of E, expression of Aqp-1 but not Aqp-5 or 7 was markedly elevated in the inner stroma and myometrium. The stromal and myometrial Aqp might indicate distribution of this protein in the blood vessels. The findings were supported by previous studies in mice, which showed that under E influence, Aqp-1 was highly
expressed in the myometrial (Jablonski et al., 2003) and in the stromal blood vessels (Richard et al., 2003). The further report indicates that E induced shifting of Aqp-1 to the blood vessels in the stroma (Richard et al., 2003). In addition to stroma and myometrium, I have found that E also causes high expression of Aqp-1 in the epithelium lining the uterine lumen. High epithelial Aqp-1 expression indicated that this channel might be involved in mediating the H₂O imbibition process into the uterine lumen.

Besides E, Aqp-1 expression was also found to be enhanced by other sex hormones. In this study, I have shown that T could increase Aqp-1 expression in the uterus. P could also enhance expression of uterine Aqp-1. Both E and P have been shown to influence uterine Aqp-1 expression as this isoform expression in the pig uterus was reported to increase throughout the oestrus cycle (Skowronska, 2010). Meanwhile, studies in rats indicated that P was able to induce redistribution of Aqp-1 to the stromal endothelial cells and to the inner circular layer of the myometrium (Lindsay & Murphy, 2006). While the P effect was thought to be important in the development of stromal edema at the implantation site (Huang et al., 2006), the effect of this hormone was also proposed to participate in the fluid loss from the uterine lumen (Lindsay & Murphy, 2006).

In this study, levels of Aqp-1 expression were moderately increased under T influence. However, the pattern of Aqp-1 distribution in the uterus was slightly different from that observed under E. Under T influence, Aqp-1 was found to be distributed mostly in the epithelia with lesser distribution in stroma and myometrium. The different distribution pattern indicates different roles of Aqp-1. For examples, under T influence, Aqp-1 might participate in the uterine fluid absorption resulting in luminal fluid loss, however, under E influence, high stromal distribution suggested that this channel might
participate in the fluid imbibition process into the uterine lumen. Similar effects were observed in rats receiving E followed by T treatment.

In these rats, levels of Aqp-1 were found to be higher compared to rats receiving E followed by vehicle treatment with this protein mostly distributed in luminal epithelium. It was further shown that E-sensitized uteri, T effect on uterine Aqp-1 was markedly lesser compared to the T effect in non-E-treated uteri. When E and T were concomitantly given, levels of Aqp-1 expression were markedly lower compared to E-only treatment. The reasons are being unknown, however, there was a possibility that T could increase redistribution of this channel to the epithelium, while decreases the expression levels of this channel in the stroma and myometrium. T effect was supported by the observation in post-menopausal uteri. Under this condition, high T level was found to be associated with increased expression of androgen receptor in endometrium (Zang et al., 2008) and vagina (Traish et al., 2007). This could explain the increased uterine responsiveness toward androgen in the state of sex-steroid deficient. There was a possibility that pre-exposure to E could lead to decreased tissue sensitivity to T in view that E was reported to down regulate androgen-receptor expression (Stover et al., 1987).

It was found that expression of Aqp-5 in the uterus was enhanced by T and was diminished following E treatment. Under T influence, Aqp-5 was localized to the apical and basolateral membranes of luminal epithelium and stroma. These findings raised the possibility that Aqp-5 might participate in fluid reabsorption from the uterine lumen under T influence. Previous studies showed that Aqp-5 expression in the uterus was enhanced by P (Lindsay & Murphy, 2006) and at the time of embryo implantation, when P level was high (Richard et al., 2003). Enhanced expression of Aqp-5 has been proposed to mediate P-induced uterine fluid reabsorption, where similar mechanism
might occur in the uterus under T influence. In this study, levels of Aqp-7 expression were elevated under T influence, while E had no effect on the expression of this protein channel. This finding was supported by the report that uterine Aqp-7 expression increases after ovulation, at the time when T level is high and this increase might be essential for decidualisation (Peng et al., 2011; Klein et al., 2013). The involvement of Aqp-7 in T-induced decidualisation, therefore, warrants further investigation. Besides T, P was also reported to stimulate increased in Aqp-7 expression. In non-pregnant mares, high expression of Aqp-7 eight days after ovulation coincides with the high level of circulating P (Klein et al., 2013) indicating that this isoform might be involved in P-induced uterine fluid reabsorption. It was hypothesized that similar effects might occur under T in which T-induced up-regulation of Aqp-7 in uterine luminal epithelium could mediate the efflux of water from the uterine lumen.

It was concluded that T administration decreased uterine luminal fluid volume, chloride concentration, cAMP level as well as down-regulated CFTR expression, but up-regulated the expression of Aqp-1, 5 and 7, which may play a role in mediating the adverse effect of T on fertility in females.
CHAPTER 5: EFFECTS OF TESTOSTERONE ON ENDOMETRIAL RECEPIVITY IN OVARIECTOMISED RATS RECEIVING SEX STEROID REPLACEMENT MIMICKING HORMONAL CHANGES IN EARLY PREGNANCY

5.1 Background

Implantation is a highly regulated event that involves interactions between the blastocyst and the receptive endometrium. Implantation occurs during a period known as uterine receptivity (Tranguch et al., 2005). During this period, complex signal exchanges occur between embryo and endometrium (Simon et al., 2000). Uterine receptivity is precisely coordinated by ovarian steroids (Paulson, 2011). Development of uterine receptivity can be affected by several hormonal and environmental factors (Xiao et al., 2011). In females, plasma testosterone (T) levels were reported to fluctuate throughout the menstrual cycle and in early pregnancy (Concannon & Castracane, 1985). Its high levels could interfere uterine receptivity development (Diao et al., 2008). High plasma testosterone levels were also linked to infertility and were suggested to suppress ovulation (Johansson and Stener-Victorin, 2013) as well as decidualization (Gonzalez et al., 2012). Additionally, expression of genes that participates in uterine receptivity development could also be impaired (Yan et al., 2012).

Pinopodes are dome-like projections arising from the apical membrane of the uterine luminal epithelium (Martel et al., 1991). In rats, this projection appears at day 4, peaking at day 5 and rapidly decline at day 6, coincide with uterine receptivity or implantation window period. The appearance of pinopodes coincides with the expression of other uterine receptivity markers such as leukaemia inhibitory factor (LIF) (Ding et al., 2008), Muc-1, L-selectin, αvβ3 integrin and heparin-binding
epidermal growth factor (HBEGF) (Achache & Revel, 2006; Aghajanova et al., 2008). In humans, the role of pinopode as uterine receptivity marker is debatable (Quinn & Casper, 2009). In rodents, pinopodes are involved in pinocytosis which assists removal of fluid from the uterine lumen to initiate luminal closure preceding blastocyst attachment (Parr & Parr, 1982). Additionally, this projection was reported to participate in embryo-endometrial contact via L-selectin ligand, MECA-79 (Shamonki et al., 2006). In humans, pinopodes were found to release secretory vesicles containing LIF into the uterine lumen (Kabir-Salmani et al., 2005). Pinopodes expression is P-dependent (Stavreus-Evers et al., 2001). A strong correlation between pinopodes with plasma P levels and P receptor expression was reported in the human (Stavreus-Evers et al., 2001), rats (Martel et al., 1991) and mice (Rashidi et al., 2012) uterus. However, E causes loss of pinopodes from the endometrial surface (Martel et al., 1991). Ovariectomised mice were found to have a high number of pinopodes, suggesting that E removal most probably causes increased in pinopodes expression (Quinn et al., 2007).

Tight junctions (TJs) are most apical part of junctional complexes located next to the adherence junction between epithelial cells and represents the boundary between plasma membrane of two adjacent cells (Tsukita & Furuse, 2000a). The number of strands and interconnections determine the tightness of the barrier formed by the TJs network (Claude & Goodenough, 1973). In the uterus, TJs regulate the diffusion of proteins and molecules between apical and basolateral membranes. It is also involved in controlling ion and H2O transports across paracellular spaces (Tsukita & Furuse, 2000b). The composition and ratio of transmembrane proteins claudin-4 and occludin determine permeability and selectivity of TJs (Tsukita et al., 2001). Claudin-4 is the key component of TJs which polymerizes within the plasma membrane as fibrils to form TJs strand (Furuse & Tsukita, 2006; Van Itallie & Anderson, 2006; Angelow et al., 2008). Occludin is incorporated and localized close to claudin-based TJs strand (Furuse
et al., 1993; Fujimoto, 1995). Hormones can control the permeability and selectivity of TJs. Under the influence of E, TJs become parallel with fewer branches and interconnections (Murphy et al., 1982). However, P causes increased network and depth of TJs with more branches and interconnections. TJs are impermeable at the time of implantation (Murphy et al., 1982).

Several molecules play important roles in embryo implantation including \(\alpha\nu\beta3\) integrin, E-cadherin and Muc-1 (Aplin, 2000). Integrin is involved in cell-cell and cell-matrix interactions and is one of the markers of endometrial receptivity (Lessey, 2003). Integrin is formed from the association of two different, non-covalently linked subunits, \(\alpha\) and \(\beta\). Three integrin subunits that have been reported in the female reproductive tract include \(\alpha1\beta1\), \(\alpha4\beta1\), and \(\alpha\nu\beta3\) (Thomas et al., 2002). Amongst these, \(\alpha\nu\beta3\) integrin interacts with trophoblast during attachment (Apparao et al., 2001). E-cadherin plays a role in adhesion between embryos and endometrium (Achache & Revel, 2006). E-cadherin is expressed at the apical membrane of luminal epithelium and between adjacent epithelial cells (Li et al., 2002). Muc-1 is a high molecular weight glycosylated macromolecule that plays a role as an anti-adhesive agent which prevents blastocyst adhesion to the endometrium by steric hindrance phenomenon. Additionally, this molecule functions to lubricate and protects blastocyst against proteolysis and microbial attack (Brayman et al., 2004). In uteri, Muc-1 is expressed at the apical surface of luminal and glandular epithelia. Down-regulation of Muc-1 at the implantation site removes the barrier between blastocyst and endometrial, allowing blastocyst to implant (Carson et al., 2000).

So far, the effects of T on pinopodes, TJs and expression of TJs protein as well as the molecules that appear during uterine receptivity period were unknown. I hypothesized that T could affect these parameters thus, interfering blastocyst implantation. This study
investigated the effects of T on pinopodes and TJs morphology and expression of MECA-79, claudin-4, occludin, αvβ3 integrin, E-cadherin and Muc-1 in sex-steroid replaced ovariectomised rats mimicking hormonal changes in early pregnancy to elucidate mechanisms underlying adverse T effect on blastocyst implantation.
5.2 Results

5.2.1 Pinopodes and L-selectin ligand (MECA-79) distribution and expression.

5.2.1.1 Field Emission Scanning Electron Microscopy (FESEM) images of endometrial pinopodes

Figure 5.1: Field emission scanning electron microscopy (FESEM) of the endometrium in different experimental groups. Abundant bleb-like projections were seen in rats receiving normal steroid-replacement regime. Fewer pinopodes were seen following administration of T during uterine receptivity period which were reverted by flutamide but not finasteride. C: control, N: normal steroid replacement regime, T: testosterone only injection with normal steroid replacement regime, T + FLU: testosterone and flutamide administration with normal steroid replacement regime and T + FIN: testosterone and finasteride administration with normal steroid replacement regime. NP: normal early pregnant rat. Arrow pointing toward pinopodes. Scale bar: 40 µm. n=3 per treatment group.
Figure 5.1 shows FESEM images of the endometrium in rats receiving peanut oil only (control), normal steroid replacement regime, normal steroid replacement regime with testosterone (T) injection (days 6-8) and treatment with normal steroid replacement regime with testosterone and flutamide (T+FLU) or testosterone and finasteride (T+FIN) (days 6-8). Our findings showed abundant pinopodes were expressed at the apical surface of the luminal epithelium in rats receiving a normal sex-steroid replacement. Injection of T from days 6-8 resulted in reduced pinopode expression compared to the rats receiving normal sex-steroid replacement without T. Meanwhile, concomitant injection of T+FLU from days 6-8 of the steroid replacement regime resulted in an increased number of pinopodes at the endometrial surface compared to T-only treatment. Concomitant T+FIN treatment from days 6-8 however did not cause any significant changes in pinopodes when compared to T only treatment.
5.2.1.2 Transmission Electron Microscopy (TEM) images of endometrial pinopodes

Figure 5.2: Transmission electron microscopy (TEM) of endometrium in different experimental rats. Pinopode projections were seen in rats which received normal steroid-replacement regime. Apical membrane with nearly absent pinopodes was seen following T administration during uterine receptivity period which, were reverted by flutamide but not finasteride. P= pinopode. C: control, N: normal steroid replacement regime, T: testosterone only injection with normal steroid replacement regime, T + FLU: testosterone and flutamide administration with normal steroid replacement regime and T+FIN: testosterone and finasteride administration with normal steroid replacement regime. NP: normal early pregnant rat. Arrow pointing toward pinopodes. Scale bar: 1µm. n=3 per treatment group.

Figure 5.2 shows TEM images of the endometrium of rats receiving peanut oil (control), normal steroid replacement, normal steroid replacement with T injection (days 6-8) and normal steroid replacement with T+FLU or T+FIN (days 6-8). Pinopodes were seen at the apical membrane of endometrial epithelium in rats receiving normal steroid replacement. T injection from days 6-8 resulted in near disappearance of pinopodes with only microvilli was seen. Concomitant administration of T+FLU from days 6-8 of the normal steroid replacement regime caused the appearance of pinopodes
at the apical surface. Meanwhile, fewer pinopodes appear in rats receiving T+FIN from days 6-8 which were almost similar to the effect of T-only treatment.

5.2.1.3 MECA-79 protein distribution

Figure 5.3: MECA-79 protein distribution at the apical membrane of the endometrial luminal epithelium in different experimental rats. MECA-79 was seen to be distributed at apical membrane of luminal epithelium predominantly in rats receiving normal steroid replacement and in rats receiving concomitant T+FLU injection during uterine receptivity period. C: control, N: normal steroid replacement regime, T: testosterone only injection with normal steroid replacement regime, T + FLU: testosterone and flutamide administration with normal steroid replacement regime and T + FIN: testosterone and finasteride administration with normal steroid replacement regime, NC: negative control. L: lumen. Arrow pointing toward MECA-79. Scale bar: 50 µm. n=6 per treatment group.

Figure 5.3 shows immunohistochemistry images of MECA-79 in different experimental rats. The higher staining intensity was observed in rats receiving normal steroid replacement. The staining intensity was markedly reduced following T administration from days 6-8 of the normal steroid replacement regime. Concomitant
administration of T+FLU from days 6-8 caused an increase in immunostaining intensity, however, no difference in intensity was noted following T+FIN treatment and T-only treatment.

5.2.1.4 MECA-79 protein expression

Figure 5.4: Immunoblotting analysis of MECA-79 protein in different experimental rats. (a) Immunoblot image of protein band in different experimental group (b) analyses of the intensity of protein bands in different experimental rats. *p<0.05 as compared to normal steroid replacement, †p<0.05 as compared with T only treatment. C: control, N: normal steroid replacement regime, T: testosterone only injection with normal steroid replacement regime, T + FLU: testosterone and flutamide administration with normal steroid replacement regime and T + FIN: testosterone and finasteride administration with normal steroid replacement regime. n = 6 per treatment group. Data presented as mean ± SEM.

Figure 5.4 (a) shows representative image of Western blot band and (b) shows analyses of protein band density of uterine tissue homogenate following incubation with MECA-79 antibody in rats treated with peanut oil (control), normal steroid replacement...
regime, normal steroid replacement regime with T (days 6-8) and normal steroid replacement regime with T+FLU or T+FIN (days 6-8). These findings indicated that the density of MECA-79 protein band was highest in rats receiving normal steroid replacement regime and lowest following T-only treatment from days 6-8. Co-administration of T+FLU from days 6-8 resulted in a significant increase in protein band density. Meanwhile, no significant difference was noted between concomitant injection of T+FIN when compared to T only treatment.
5.2.2 Tight junction, claudin-4 and occludin distribution and expression

5.2.2.1 Morphology of tight junction

![Figure 5.5: Ultramorphological appearance of uterine tight junction. Transmission electron microscopy (TEM) images showing tight junction morphology in the endometrium of different experimental groups. C: vehicle control, N: normal sex-steroid replacement regime, T: testosterone only injection, T + FLU: testosterone and flutamide injection, T + FIN: testosterone and finasteride injection, NP: normal early pregnant rat. TJ: tight junction, M: microvilli, V: vacuole. Arrow pointing toward pinopodes. Scale bar: 1 µm. n=3 per treatment group.]

TEM images of endometrium showed geometrically complex and interconnected tight junction strands were seen in the endometrium of rats receiving a normal sex-steroid replacement (Figure 5.5). T administration between days 6-8 resulted in TJs
strand to become parallel. Concomitant administration of T+FLU or T+FIN did not cause changes to the appearance of tight junction when compared to T-only treatment.

5.2.2.2 Claudin-4 protein distribution

**Figure 5.6:** Distribution of claudin-4 in uterus. The site of expression of claudin-4 protein was indicated by bright fluorescence signals. C: vehicle control, N: normal steroid replacement regime, T: testosterone only injection, T + FLU: testosterone and flutamide injection. T + FIN: testosterone and finasteride injection. NC: negative control. L = lumen. Arrows pointing towards claudin-4. Scale bar = 50 µm. n=6 per treatment group.

Claudin-4 protein could be seen distributed at the apical membrane of luminal epithelium. Immunofluorescence images of uterus showed the highest distribution of claudin-4 in rats receiving sex-steroid treatment without T (Figure 5.6). Claudin-4 distribution was lower in rats receiving T-only treatment between days 6-8.
Concomitant administration of T+FLU and T+FIN also caused low claudin-4 distribution.

5.2.2.3 Claudin-4 mRNA expression

**Figure 5.7:** Levels of claudin-4 mRNA in uterus. Highest claudin-4 mRNA levels were observed in rats received normal sex-steroid replacement regime. In these rats, levels of claudin-4 mRNA were markedly reduced following T injection (days 6-8). C: vehicle control, N: normal sex-steroid replacement regime, T: testosterone only injection, T + FLU: testosterone and flutamide injection, T + FIN: testosterone and finasteride injection. *p<0.05 compared to normal sex-steroid replacement regime. n=6 per treatment group. Data presented as mean±SEM.

Levels of claudin-4 mRNA in the uterus were highest in rats receiving normal sex-steroid replacement (~11 folds higher than control) (Figure 5.7). Administration of T between days 6 to 8 resulted in claudin-4 mRNA levels decrease (p<0.05). No significant changes in claudin-4 mRNA levels were observed in rats receiving T-only treatment compared to rats receiving T+FLU or T+FIN treatment.
5.2.2.4 Occludin protein distribution

![Diagram showing occludin protein distribution in the uterus](image)

**Figure 5.8:** Distribution of occludin in uterus. The site of expression of occludin protein was indicated by bright fluorescence signals. C: vehicle control, N: normal steroid replacement regime, T: testosterone only injection, T + FLU: testosterone and flutamide injection. T + FIN: testosterone and finasteride injection. NC: negative control. L = lumen. Arrows pointing towards claudin-4. Scale bar = 50 µm. n=6 per treatment group.

Occludin protein was seen distributed at the apical membrane of uterine luminal epithelium. Distribution of occludin in the uterus was higher in rats receiving a sex-steroid replacement without T (Figure 5.8). In these rats, distribution of occludin was lower following treatment with T between days 6-8. Low occludin distribution was also observed in rats that received T+FLU and T+FIN treatments.
5.2.2.5 Occludin mRNA expression

Figure 5.9: Levels of occludin mRNA in uterus. Highest occludin mRNA was observed in rats receiving normal sex-steroid replacement regime. T administration between days 6-8 resulted in markedly reduced occludin mRNA levels. C: vehicle control, N: normal sex-steroid replacement regime, T: testosterone only injection, T + FLU: testosterone and flutamide injection. T + FIN: testosterone and finasteride injection. *p<0.05 as compared to normal sex-steroid replacement regime. n=6 per treatment group. Data presented as mean±SEM.

Levels of occludin mRNA in the uterus were highest in rats receiving normal sex-steroid replacement (Figure 5.9). Administration of T between days 6 to 8 resulted in claudin-4 mRNA level to decrease (p<0.05). No significant changes in occludin mRNA levels were observed among rats receiving T-only treatment and receiving T+FLU or T+FIN treatment.
5.2.3 αvβ3 integrin, E-cadherin and Muc-1 distribution and expression

5.2.3.1 αvβ3 integrin protein distribution

Figure 5.10: Distribution of αvβ3 integrin in uterus. Immunofluorescence images showing high distribution of αvβ3 integrin in luminal epithelium of rats which received normal steroid replacement. αvβ3 integrin level was markedly reduced following T injection between days 6-8. C: vehicle control, N: normal steroid replacement regime, T: testosterone only injection, T + FLU: testosterone and flutamide and T + FIN: testosterone and finasteride. NC: negative control. Arrows pointing towards αvβ3 integrin distribution. L = lumen. Scale bar = 50 µm. n=6 per treatment group.

αvβ3 integrin protein distribution could be seen at the apical membrane of luminal epithelium. Highest distribution of αvβ3 integrin in the uterus was observed in rats receiving normal sex-steroid regime without T (Figure 5.10). In rats that received T between days 6-8, levels of αvβ3 integrin was markedly reduced. Concomitant administration of T+FLU or T+FIN between days 6-8 cause no changes in αvβ3 integrin levels in uterus compared to T only treatment.
5.2.3.2 αvβ3 integrin mRNA expression

Figure 5.11: αvβ3 integrin mRNA level in uterus. Marked increase in αvβ3 integrin mRNA level was observed in rats received normal steroid replacement. Significant decrease in αvβ3 integrin mRNA level was observed in rats receiving T treatment between days 6-8 with and without flutamide or finasteride. C: vehicle control, N: normal steroid replacement, T: testosterone only injection, T + FLU: testosterone and flutamide injection, T + FIN: testosterone and finasteride injection. *p< 0.05 compared to normal steroid replacement. n=6 per treatment group. Data presented as mean±SEM.

Levels of αvβ3 integrin mRNA was highest in rats that received normal sex-steroid replacement regime (5.2 folds higher than control) (Figure 5.11). T injection between days 6 to 8 resulted in decreased αvβ3 integrin mRNA level compared to normal control rats. Concomitant administration of T+FLU and T+FIN did not cause significant changes in the level of αvβ3 integrin mRNA in uterus when compared to T treatment.
5.2.3.3 E-cadherin protein distribution

![Immunofluorescence images showing high distribution of E-cadherin in luminal epithelium of rats which received normal steroid replacement. E-cadherin level was markedly reduced following T injection between days 6-8. C: vehicle control, N: normal steroid replacement regime, T: testosterone only injection, T + FLU: testosterone and flutamide and T + FIN: testosterone and finasteride. NC: negative control. Arrows pointing towards E-cadherin distribution. L = lumen. Scale bar = 50 µm. n=6 per treatment group.](image)

**Figure 5.12:** Distribution E-cadherin in uterus. Immunofluorescence images showing high distribution of E-cadherin in luminal epithelium of rats which received normal steroid replacement. E-cadherin level was markedly reduced following T injection between days 6-8. C: vehicle control, N: normal steroid replacement regime, T: testosterone only injection, T + FLU: testosterone and flutamide and T + FIN: testosterone and finasteride. NC: negative control. Arrows pointing towards E-cadherin distribution. L = lumen. Scale bar = 50 µm. n=6 per treatment group.

E-cadherin protein was seen distributed at the apical membrane of luminal epithelium. Distribution of E-cadherin in uterus was highest in rats receiving normal sex-steroid replacement regime without T (Figure 5.12). E-cadherin levels were markedly reduced following T treatment between days 6-8. However, concomitant administration of T+FLU and T+FIN resulted in no changes in E-cadherin levels in uterus compared to T-only treatment.
5.2.3.4 E-cadherin mRNA expression

Figure 5.13: E-cadherin mRNA level in uterus. E-cadherin mRNA level was highest in rats receiving normal sex-steroid replacement. E-cadherin mRNA levels were significantly decreased in rats receiving T treatment with and without flutamide or finasteride. C: vehicle control, N: normal steroid replacement regime, T: testosterone only injection, T + FLU: testosterone and flutamide injection and T + FIN: testosterone and finasteride injection. *p < 0.05 compared to normal steroid replacement. n=6 per treatment group. Data presented as mean±SEM.

E-cadherin mRNA levels were highest in rats receiving normal sex-steroid replacement regime (two folds higher than control) (Figure 5.13). A markedly reduced mRNA level was observed in rats receiving T between days 6 to 8. Meanwhile, no significant differences in E-cadherin mRNA levels were seen between T+FLU and T+FIN treated rats.
5.2.3.5 Mucin-1 protein distribution

**Figure 5.14**: Distribution of Muc-1 in uterus. Immunofluorescence images showing distribution of Muc-1 which was highest in luminal epithelia of rats receiving normal sex-steroid replacement. Low Muc-1 distribution was observed following T treatment between days 6 - 8. C: vehicle control, N: normal sex-steroid replacement, T: testosterone only injection, T + FLU: testosterone and flutamide injection and T + FIN: testosterone and finasteride injection. NC: negative control. Arrows pointing towards Muc-1 distribution. L = lumen. Scale bar = 50 µm. n=6 per treatment group.

Muc-1 protein was seen to be distributed at the apical membrane of luminal epithelium. Distribution of Muc-1 protein was highest in rats receiving normal sex-steroid replacement without T (Figure 5.14). Mucin-1 levels were markedly reduced in rats receiving T-only treatment between days 6-8. Meanwhile administration of T+FLU and T+FIN between days 6-8 did not cause changes in Muc-1 levels compared to T only treatment.
5.2.3.6 Mucin-1 mRNA expression

![Mucin-1 mRNA expression diagram]

**Figure 5.15**: Muc-1 mRNA level in uterus. Muc-1 mRNA level was highest in rats receiving normal sex-steroid replacement. Muc-1 mRNA levels were significantly decreased in rats receiving T treatment with and without flutamide or finasteride. C: vehicle control, N: normal steroid replacement regime, T: testosterone only injection, T + FLU: testosterone and flutamide injection and T + FIN: testosterone and finasteride injection. *p < 0.05 compared to normal steroid replacement. n=6 per treatment group. Data presented as mean±SEM.

Figure 5.15 showed Muc-1 mRNA level were highest in rats receiving normal sex-steroid replacement (1.5 fold higher than control). T administration between days 6 to 8 resulted in a significant decrease in Muc-1 mRNA levels whilst no changes were noted following combined T+FLU and T+FIN treatment when compared to T-only treatment.
5.3 Discussion

This study investigates for the first time effect of T on pinopode expression in rats during uterine receptivity period. In this study, the followings were observed: (i) T administration from days 6-8 (uterine receptivity period) caused significant decrease in pinopode expression at apical surface of the luminal epithelium, (ii) T administration (days 6-8) resulted in decreased expression of MECA-79 at apical surface of uterine luminal epithelium (iii) T effect was antagonized by flutamide, which confirmed androgen receptor involvement and (iv) T effects were not antagonized by finasteride, indicating that 5α-dihydrotestosterone (DHT) was not involved in causing these effects.

This study has shown that in rats receiving normal steroid replacement regime without T, pinopodes appeared moderately wrinkled with oblong shaped structure protruding from the apical membrane of luminal epithelium. These morphological appearances were consistent with the previous reports (Enders & Nelson, 1973; Parr & Parr, 1974). In addition, pinopodes were seen to project above the level of microvilli and contain multiple vacuoles. Several larger vacuoles were also seen at the base of these projections. These vacuoles were thought to be involved in endocytosis which helps to remove the uterine luminal fluid to initiate uterine closure (Nilsson, 1972; Parr & Parr, 1974). In rats, uterine closure event is important to help to sandwich the implanting the blastocyst between the two opposing endometrial surfaces, initiating the first physical contact between a blastocyst and the receptive endometrium. Parr (1974) reported that pinopodes also contain actin-rich stalk which appears as bands on the pedicle. Meanwhile, it was estimated that in rats, between 5.5% to 20% of the endometrial epithelial cells were covered with pinopodes during uterine receptivity period that might help to generate sufficient force to remove the fluid from uterine the lumen (Quinn et al., 2007).
In view that pinopodes are P-dependent (Singh et al., 1996), the appearance of these projections were expected following this hormonal treatment. Concomitant administration of T resulted in a near absent of pinopodes from the endometrial surface. This study has shown that 5α-DHT was not involved in inhibiting uterine pinopodes expression as evidence of lack of finasteride inhibition of T-mediated decrease in pinopodes expression. Uterine tissue was reported to have a higher affinity towards T than 5α-DHT (Giannopoulous, 1973). However, several uterine effects of T have been shown to be mediated via 5α-DHT, examples expression of oestrogen receptor (ER) (Cárdenas & Pope, 2005) and uterine growth (Zhang et al., 2004). Additionally, 5α-DHT is also important during the secretory phase of the cycle as AR and 5α-reductase enzyme levels in the uterus were reported highest during this period (Ito et al., 2002).

L-selectin ligand (MECA-79) mediates blastocyst-endometrial contact prior to blastocyst attachment. This study has shown that MECA-79 expression was significantly higher in the endometrium of ovariectomised rats, which received normal steroid replacement regime without T. This protein was expressed mainly at the apical membrane of the uterine luminal epithelium, consistent with its role in embryo attachment to the receptive endometrium. In humans, L-selectin ligand was up-regulated from the day of ovulation to day 6-post ovulation (Shamonki et al., 2006) with peak expression in the mid-luteal phase of the cycle (Lai et al., 2005). In addition, endometrial MECA-79 expression was reported higher in fertile than infertile humans (Margarit et al., 2009). In this study, marked decrease in MECA-79 expression at the apical membrane of rat endometrial epithelia during uterine receptivity period (days 6-8) by T treatment indicated that expression of this protein was inhibited by T. In parallel with the effect on pinopodes expression, the T effect on MECA-79 expression was also inhibited by flutamide, indicating the involvement of AR-mediated the effects. Meanwhile, 5α-DHT has no role in MECA-79 expression as evidence from the lack of
inhibition by 5α-reductase inhibitor on the T-mediated suppression of MECA-79 expression in the uterus during the receptive period.

In this study, I also made the following observations (i) co-administration of T with sex-steroids between days 6 to 8 resulted in loss of complexity of TJs and, reduces the expression of TJs protein i.e. claudin-4 and occludin and adhesion molecules i.e. αβ3 integrin, E-cadherin and mucin-1 and (ii) effects of T not mediated via AR or 5α-DHT, an active T metabolite.

Under normal conditions, geometrically complex TJs appearance was due to the effect of P (Murphy et al., 1981). TJs play an important role in controlling the diffusion of fluid and molecules through the paracellular pathway (Murphy et al., 1981, 1982). Its permeability can be affected by the levels of its integral proteins i.e. claudin-4 and occludin. Under the influence of P, “tight” TJs are formed which able to prevent diffusion of molecules (Murphy et al., 1981). Claudin-4 plays an important role in membrane to membrane adhesion and also form a diffusion barrier (Tsukita et al., 2001) while occludin help to regulate TJs permeability (Orchard & Murphy, 2002). Decreased in TJs complexity and down-regulated expression of claudin-4 and occludin under the influence of T could result in “leaky” TJs that allow movement of fluid and ions through the paracellular pathway (Capaldo & Nusrat, 2009). Therefore, T could potentially interfere with fluid and ionic movements and cell adhesion in the early pregnant uterus.

Besides affecting TJs and levels of expression of the related proteins, levels of adhesion molecules such as αβ3 integrin, E-cadherin and Muc-1 were also significantly decreased following T treatment. αβ3 integrin is involved in the maternal recognition of embryo (Reddy et al., 2001). Expression of αβ3 integrin was markedly reduced in infertile women indicating that this molecule plays an important role in early
pregnancy establishment (Lessey, 1998). Expression of $\alpha v \beta 3$ integrin was reported high under the influence of P (Srinivasan et al., 2009). E-cadherin also plays important role in embryonic communication with uterus and is involve in trophoblast invasion (Liu et al., 2006). P is known to up-regulate E-cadherin in the uterus (Jha et al., 2006). Possible involvement of P was supported by the observation that expression of E-cadherin in early pregnant rats was high during dioestrus stage when P levels were highest (Slater et al., 2002). Under the influence of T, low expression of E-cadherin might interfere with interactions between the embryo and the endometrium.

The study has shown that expression of Muc-1 was markedly reduced in rats that received T treatment between days 6-8 of the sex-steroid replacement regime. Muc-1 was highly expressed in uterus under the influence of P (Aplin et al., 1998). High expression of Muc-1 was reported at the apical membrane of luminal epithelium, which helps to prevent adhesion between the blastocyst to endometrial epithelium (Horne et al., 2002). Muc-1 forms scaffold that allows the blastocyst to roll over the surface, preventing it from sticking to the site other than that for implantation (Giudice, 1999). Reduced expression of Muc-1 under T influence could result in premature attachment of embryo that might cause implantation failure.

It was observed that effects of T on TJ and expression of TJ proteins and adhesion molecules did not involve AR. Additionally, these effects were also not mediated by DHT, an active T metabolite. There were possibilities that T exert its effect through non-genomic pathway as androgens were shown to exert non-genomic effects on uterine smooth muscle (Perusquía et al., 1990). Other sex-steroids including P was also reported to partly mediate its effect through the non-genomic pathway (Gellersen et al., 2009).
This study has an important contribution towards understanding the mechanism underlying T effect on blastocyst implantation as blastocyst fails to implant in a situation where T level was high for example in polycystic ovarian disease (PCO) (Choi et al., 2012). With high rate of implantation failure even after ovulation induction, the reduced fertility rate in PCO patient was not only due to anovulation however could also be due to endometrial dysfunction (Shang et al., 2012). In PCO, other biomarkers of endometrial receptivity such as αvβ3 integrin and glycodelin were also significantly decreased, while epithelial expression of ER alpha (ERα) abnormally persists throughout the implantation window period. Additionally, stromal decidualisation was also inhibited by the high insulin level (Giudice, 2006) while expression of androgen receptor in the uterus was highly elevated in this condition (Apparao et al., 2002).

In conclusion, this study has shown that T administration during uterine receptivity period in ovariectomised rats receiving sex-steroid replacement reduced pinopodes development and MECA-79 expression, the complexity of tight junction and tight junction protein expression including claudin-4 and occludin as well as reduced the expression of Muc-1, E-cadherin and αvβ3 integrin.
CHAPTER 6: EFFECTS OF TESTOSTERONE ON ENDOMETRIAL RECEPTIVITY AND EMBRYO IMPLANTATION IN INTACT EARLY PREGNANT RATS

6.1 Background

Implantation is a unique phenomenon that involves physical and physiological interactions between the embryo and receptive endometrium (Lim & Dey, 2009). Successful implantation requires a transformation of the uterus into the receptive state, in which it is ready to accept the implanting blastocyst (Aghajanova et al., 2008). Uterine receptivity is primarily coordinated by ovarian steroids i.e. 17β-oestradiol (E) and progesterone (P) (Paulson, 2011) and is associated with the appearance of specific molecules and characteristic changes in uterine ultrastructure (Aghajanova et al., 2008). The specific ultrastructural changes include pinopodes while specific molecules expressed include L-selectin ligand (MECA-79), αvβ3 integrin, E-cadherin and mucin-1 (Muc-1) (Ruijter-Villani & Stout, 2015). Tight junctions’ protein, claudin-4 and occludin serve as markers of uterine receptivity (Orchard & Murphy, 2002). Meanwhile, signalling molecules that mediate the implantation process are muscle-segment homeobox-1 (Msx-1) (Cha et al., 2015), fibroblast growth factor-1 (Fgf-1) (Sak et al., 2013) and Indian hedgehog (Ihh) (Wakitani et al., 2008).

Pinopodes are smooth mushroom-like projections arise from the apical surface of the endometrium are important microstructural markers of endometrial receptivity (Nikas, 1999). This projection was reported to participate in embryo-endometrial interaction via L-selectin ligand (Nejatbakhsh et al., 2012) that consists of an epitope, MECA-79 (Shamonki et al., 2006). Tight junctions (TJs) regulate the diffusion of protein and molecules between apical and basolateral membranes and are involved in controlling
ion, water and molecular transport across paracellular space between epithelial cells (Tsukita & Furuse, 2000b). The composition and ratio of transmembrane proteins claudin-4 and occludin contributes towards permeability and selectivity of TJs (Tsukita et al., 2001). Endometrial αvβ3 integrin interacts with trophoblast (Apparao et al., 2001) and plays important role as a receptor for embryo attachment (Lessey, 2003). E-cadherin is expressed in peri-implantation embryo and uterine epithelial cells and participated in focal contacts between trophectoderm and uterine epithelium during adhesion (Liu et al., 2006). Muc-1 is an anti-adhesive molecule that functions as a physical barrier for uterine adhesion. The removal of Muc-1 is needed for the embryo to access the apical surface of uterine epithelium (Singh & Aplin, 2009; van Mourik et al., 2009). Endometrial signalling molecules such as Ihh mediate the effect of P on stromal cell proliferation while Fgf-1 promotes epithelial proliferation by E and facilitate establishment and maintenance of pregnancy (Li et al., 2011). Msx-1 plays a role in altering epithelial cell polarity that is required for blastocyst attachment to the epithelium.

T has been reported to disturb the prostaglandin system that is involved in peri-implantation uterine development (Diao et al., 2008). T also found to suppress the expression of HOXA-10, a signalling molecule necessary for the development of uterine receptivity (Cermik et al., 2003). So far, effects of T on embryo implantation and ultrastructural changes in the uterus have never been studied. Effects of this hormone on the expression of implantation molecules such as MECA-79, αvβ3 integrin, Muc-1 and E-cadherin, tight junctional proteins i.e. claudin and occludin and signalling molecules i.e. Msx-1, Fgf-1 and Ihh have yet to be elucidated.

The aims of this part of the study were to investigate changes in the ultrastructure of the endometrium, expression of tight junctional proteins, implantation molecules and
uterine signalling molecules and to investigate the rate of embryo implantation following T treatment to intact early pregnant rats beginning from the first day of pregnancy. These findings were important to elucidate effects and mechanisms underlying T effects on embryo implantation in intact early pregnancy females.
6.2 Results

6.2.1 Plasma testosterone, 17β-oestradiol, progesterone, LH and FSH levels

Table 6.1: Serum level of reproductive hormones at day 4 of pregnancy. C: normal pregnant, T250: 250µg/kg/day testosterone and T500: 500µg/kg/day testosterone. *p<0.05 compared to C. n=6 per treatment group. Data presented as mean±SEM

<table>
<thead>
<tr>
<th>HORMONE</th>
<th>TREATMENT</th>
<th>CONCENTRATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>17β-oestradiol</td>
<td>C</td>
<td>49.65±3.66 pmol/l</td>
</tr>
<tr>
<td></td>
<td>T250</td>
<td>*83.35±2.91 pmol/l</td>
</tr>
<tr>
<td></td>
<td>T500</td>
<td>*102.67±1.76 pmol/l</td>
</tr>
<tr>
<td>Progesterone</td>
<td>C</td>
<td>166.93±8.71 nmol/l</td>
</tr>
<tr>
<td></td>
<td>T250</td>
<td>134.8±12.20 nmol/l</td>
</tr>
<tr>
<td></td>
<td>T500</td>
<td>161.73±3.98 nmol/l</td>
</tr>
<tr>
<td>Testosterone</td>
<td>C</td>
<td>0.60±0.2 nmol/l</td>
</tr>
<tr>
<td></td>
<td>T250</td>
<td>*5.90±1.44 nmol/l</td>
</tr>
<tr>
<td></td>
<td>T500</td>
<td>*30.07±1.47 nmol/l</td>
</tr>
<tr>
<td>LH</td>
<td>C</td>
<td>0.1 IU/l</td>
</tr>
<tr>
<td></td>
<td>T250</td>
<td>0.1 IU/l</td>
</tr>
<tr>
<td></td>
<td>T500</td>
<td>0.1 IU/l</td>
</tr>
<tr>
<td>FSH</td>
<td>C</td>
<td>0.3 IU/l</td>
</tr>
<tr>
<td></td>
<td>T250</td>
<td>0.3 IU/l</td>
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<tr>
<td></td>
<td>T500</td>
<td>0.3 IU/l</td>
</tr>
</tbody>
</table>

Plasma level of E at day 4 of pregnancy markedly increased following administration of T (Table 6.1). The increase was dose-dependent. However, plasma level of P in the control group did not differ significantly with T-treated group. Meanwhile, plasma levels of T increased with increasing dose of subcutaneously administered testosterone. Following administration of T at 500µg/kg/day, plasma levels of this hormone were approximately 50 folds higher than control. No significant difference in LH and FSH levels were noted between control and T-treated rats.
6.2.2 Number of embryo implantation sites

The highest number of implantation site could be seen in normal pregnant rats not treated with T (Figure 6.1). A significant decrease in the number of implantation site could be seen in rats receiving T at 500µg/kg/day. In normal pregnant rats, the mean number implantation site was 10 while only 2 sites were detected in T500 group. In rats receiving T at 250µg/kg/day, the decrease in the number of implantation site was not significant compared to rats not receiving T treatment.

Figure 6.1: Implantation site at day 6 of pregnancy. (A) Arrows showing blue spots in uterus where embryo implantation occurs (B) analyses of the number of implantation site in rats receiving different treatment. C: normal pregnant rats, T250: 250µg/kg/day testosterone and T500: 500µg/kg/day testosterone. *p<0.05 compared to C. n=6 per treatment group. Data presented as mean±SEM
6.2.3 Ultramorphological changes in endometrium

Pinopodes were seen in normal pregnant rats not receiving T treatment (Figure 6.2A). Similarly, pinopodes were also seen in rats receiving T at 250µg/kg/rat. However, in rats, which received T at 500µg/kg/day, pinopodes were not seen at the apical membrane. In these rats, only microvilli could be seen. Meanwhile, in pregnant rats not receiving T treatment, geometrically complex and interconnected strand of tight junctions (TJs) could be seen at day 4 of pregnancy (Figure 6.2B). The TJs appear complex and interconnected in rats receiving T at 250µg/kg/day. However, in rats that received T at 500µg/kg/rat, TJs were less complex and parallel.

Figure 6.2: Transmission electron microscopy (TEM) images of endometrium showing (A) pinopodes and microvilli projecting from endometrial surface (B) appearances of tight junction in endometrium. Images were taken at day 4 of pregnancy following 3 days of T treatment. P: pinopode, TJ: Tight Junctions, C: normal pregnant rats, T250: 250µg/kg/rat testosterone and T500: 500µg/kg/rat testosterone. Scale bar = 1µm. n=3 per treatment group.
6.2.4 MECA-79 expression and distribution

MECA-79 protein could be seen distributed at apical membrane of luminal epithelium. The levels of MECA-79 were highest in rats not receiving T treatment (Figure 6.3). In these rats, treatment with T at 250µg/kg/day did not cause significant changes in MECA-79 distribution compared to rats not receiving T. Meanwhile, in rats that received T at 500µg/kg/day, low MECA-79 distribution level was observed compared to rats not receiving T.

![Figure 6.3](image)

**Figure 6.3:** (A) Western blot band and expression of MECA-79 protein in uterus (B) Distribution of MECA-79 protein in uterus. Dark-brown staining depicts sites where antibody binds to proteins. L: Lumen, C: normal pregnant, T250: 250µg/kg/day testosterone and T500: 500µg/kg/day testosterone. Arrows pointing towards area of MECA-79 protein distribution. Scale bar = 100µm. *p<0.05 compared to C. n=6 per MECA-79 distribution. Data presented as mean±SEM
6.2.5 Claudin-4 expression and distribution

Figure 6.4: (A) Expression level of claudin-4 (B) distribution of claudin-4 in the uterus. Immunoblot images showing levels of expression of claudin-4 in uterus were reduced following T treatment. Fluorescence intensity for claudin-4 was highest at apical membrane of luminal epithelium of non-T treated rats. L: Lumen, C: normal pregnant, T250: 250µg/kg/rat T and T500: 500µg/kg/rat T. NC: negative control. Arrows pointing towards area of claudin-4 distribution. Scale bar = 100µm. **p<0.05 compared to C. n=6 per treatment group. Data presented as mean±SEM

Claudin-4 and was seen to be distributed at apical and basolateral membranes of luminal epithelium. Levels of claudin-4 protein were highest in pregnant rats not receiving T (Figure 6.4A). Treatment with T at 250µg/kg/day did not cause significant
changes in claudin-4 distribution levels, however the levels were significantly low in pregnant rats receiving T at 500µg/kg/day.

6.2.6 Occludin expression and distribution

Figure 6.5: (A) Expression levels of occludin (B) distribution of occludin in uterus. Immunoblot images showing levels of expression of occludin proteins in uterus were reduced following T treatment. Fluorescence intensity for claudin-4 was highest at apical membrane of luminal epithelium in non-T treated rats. L: Lumen, C: normal pregnant, T250: 250µg/kg/rat T and T500: 500µg/kg/rat T. Arrows pointing towards area of occludin distribution. Scale bar = 100µm. **p<0.05 compared to C. n=6 per treatment group. Data presented as mean±SEM
Occludin was seen to be distributed at apical and basolateral membranes of luminal epithelium. Levels of occludin protein were highest in pregnant rats not receiving T (Figure 6.5A). Treatment with T at 250µg/kg/day caused significant changes in occludin distribution levels. Marked decreases in occludin levels were seen in pregnant rats receiving T at 500µg/kg/day.

6.2.7 αvβ3 integrin expression and distribution

αvβ3 integrin was found at the apical surface of the luminal epithelium (Figure 6.6). Stromal distribution was also seen. Levels of expression of this protein were highest in pregnant rats not receiving T treatment. In these rats, treatment with T at 250µg/kg/day resulted in slightly lower distribution levels αvβ3 integrin in uterus. In rats receiving T at 500µg/kg/day, a significantly low distribution level of this protein was observed.
Figure 6.6: Expression and distribution of αvβ3 integrin in uterus. (A) Immunoblot image and analysis of intensity of αvβ3 integrin protein band (B) αvβ3 integrin protein distribution at apical membrane of endometrial luminal epithelium. The highest αvβ3 integrin protein expression and distribution were observed in pregnant rats not receiving T treatment followed by pregnant rats treated with 250μg/kg/rat T. Meanwhile, low expression and distribution were seen in rat receiving 500μg/kg/rat testosterone. L: Lumen, C: normal pregnant, T250: 250μg/kg/rat testosterone and T500: 500μg/kg/rat testosterone. Arrows pointing towards area of αvβ3 integrin distribution. Scale bar = 100μm. *p<0.05 compared to C. n=6 per treatment group. Data presented as mean±SEM.
6.2.8 E-cadherin protein expression and distribution

Figure 6.7 Expression and distribution of E-cadherin in uterus. (A) Immunoblot image and analysis of intensity of E-cadherin protein band (B) E-cadherin protein distribution in luminal epithelium. The highest E-cadherin protein expression and distribution were seen in normal pregnant group not receiving T followed by 250µg/kg/rat T-treated rats. Meanwhile, treatment with 500µg/kg/rat T resulted in lowest expression and distribution of E-cadherin. L: Lumen, C: normal pregnant, T250: 250µg/kg/rat T and T500: 500µg/kg/rat T. Arrows pointing towards the area of E-cadherin distribution. Scale bar = 100µm. *p<0.05 compared to C. n=6 per treatment group. Data presented as mean±SEM
E-cadherin was found to be distributed at the apical membrane of the luminal epithelium (Figure 6.7). Stromal distribution was also seen. Levels of expression of this protein were highest in pregnant rats not receiving T treatment. In these rats, treatment with T at 250µg/kg/day resulted in slightly lower distribution levels of this protein. In rats receiving T at 500µg/kg/day, significantly low distribution level of this protein was observed.

6.2.9 Mucin-1 protein expression and distribution

Muc-1 was found to be distributed at apical surface of luminal epithelium (Figure 6.8). Stromal distribution was also seen. The levels of expression of this protein was highest in pregnant rats not receiving T treatment. Administration of T at 250 µg/kg/rat and 500µg/kg/rat resulted in significantly low expression and apical distribution of Muc-1.
**Figure 6.8:** Expression and distribution of Muc-1 in uterus. (A) Immunoblot image and analysis of intensity of Muc-1 protein in different experimental rats (B) Muc-1 protein distribution at apical membrane of endometrial luminal epithelium in different experimental rats. Highest Muc-1 expression and distribution were observed in normal pregnant rats not receiving T treatment. L: Lumen, C: normal pregnant, T250: 250µg/kg/rat testosterone and T500: 500µg/kg/rat testosterone. Arrows pointing towards area of Muc-1 protein distribution. Scale bar = 100µm. *p<0.05 compared to C. n=6 per treatment group. Data presented as mean±SEM.
6.2.10 Msx-1, Fgf-1 and Ihh proteins expression

The highest expression of Msx-1, Fgf-1 and Ihh proteins were seen in early pregnant rats not receiving T (Figure 6.9). Expression levels of these proteins were significantly reduced following administration of T at 500µg/kg/rat. Meanwhile, in rats that received T at 250µg/kg/rat, levels of Ihh protein in uterus were slightly lower compared to the normal pregnant rats not receiving T treatment. Levels of Msx-1 and Fgf-1 proteins in rats receiving T at 250µg/kg/rat were significantly reduced compared to the normal pregnant rats not receiving T.

Figure 6.9: Expression of Msx-1, Fgf-1 and Ihh in uterus. Highest Msx-1, Fgf-1 and Ihh proteins expression were seen in pregnant rat not receiving T treatment. Meanwhile, treatment with 250µg/kg/day and 500 µg/kg/day of T resulted in decreased expression of these proteins. L: Lumen, C: normal pregnant, T250: 250µg/kg/day T and T500: 500 µg/kg/day T. *p<0.05 compared to C. n=6 per treatment group. Data presented as mean±SEM
6.3 Discussion

In this study, testosterone (T) was administered via subcutaneous injection. Subcutaneous injections would result in a stable plasma hormone levels throughout the treatment. Therefore, this would relate to a single point measurement of the hormone levels.

I have shown that the number of implantation sites was significantly reduced in rats receiving T at 500µg/kg/day, which suggest that high levels of T could suppress fertility. High levels of T could also affect ultrastructure of the endometrium and expression levels of implantation-related proteins in the uterus during receptivity period. However, administration of T at 250µg/kg/day did not cause significant changes in these parameters.

Assessment of serum hormone levels revealed that increased level of 17β-oestradiol (E) was observed in rats receiving T at 250µg/kg/day and the level further increased following injection of T at 500µg/kg/day. It is likely that T undergoes aromatization to E resulting in increased E levels with increasing doses of T (Nelson & Bulun, 2001). Evaluation of serum T levels further supported the proposed aromatization of T to E in-vivo. Following injection of T at 250µg/kg/day, levels of this hormone in plasma were significantly lower when compared to injection of T at 500µg/kg/day which was likely due to T underwent aromatization. Meanwhile, T injection did not cause significant changes in the plasma level of progesterone (P), LH and FSH.

It was seen that there was almost complete absence of pinopode in rats which received T at 500µg/kg/day while expression of MECA-79 was significantly reduced compared to rats not receiving T. Pinopodes are important microstructure that appears only during endometrial receptivity period (Nikas, 1999). Pinopodes participate in embryo-endometrial interactions through L-selectin ligand, MECA-79 (Nejatbakhsh et
The development of pinopodes in the uterus was P dependent (Martel et al., 1991). In rats, pinopodes expression correlates with the window of receptivity. It was believed that in rats, pinopodes are involved in pinocytosis of uterine fluid, which facilitates uterine closure, sandwiching the blastocyst between the opposing uterine walls. Similarly, in rats, the number of pinopodes increases at day 4 of pregnancy and becomes abundant at day 5, likely attributed to P (Psychoyos & Mandon, 1971). L-selectin ligands (MECA-79), expressed on the pinopodes surface at the time of implantation is involve in blastocyst-endometrial interaction (Genbacev OD, 2003 ). Expression of MECA-79 was higher in fertile than infertile humans suggesting that it plays a critical role in implantation (Margarit et al., 2009). The absence of MECA-79 was proposed as a reason for implantation failure (Foulk et al., 2007; Margarit et al., 2009). In view that development of pinopode and expression of MECA-79 was suppressed by T, interactions between the embryo and endometrium would be impaired, which could lead to implantation failure.

In addition to the disappearance of pinopodes, T was also found to cause decreased complexity of the TJs. In rats not receiving T, TJs appear complex with increased depth at the time of implantation (Murphy et al., 1981). TJs control the movement of ions and water and molecules through the paracellular spaces (Tsukita & Furuse, 2000b). The permeability and selectivity of TJs are controlled by claudin-4 and occludin (Tsukita et al., 2001). Therefore, reduced expression and distribution of claudin-4 and occludin would result in “leaky” TJs that allow movement of fluid through the paracellular space (Anderson & Van Itallie, 2009). Besides that, distribution of occludin was also seen in stroma of the uterus. This observation similar to the finding by Na et al., (2007) which increased stroma occludin reactivity was detected and it altered the paracellular barrier properties during receptivity period. The changes that occur following high-dose T
treatment could potentially disturb normal regulation of fluid during receptivity period that could likely interfere with embryo implantation.

It was shown that expression and distribution of αvβ3 integrin were significantly reduced following T administration. αvβ3 integrin plays important roles in embryo attachment (Lessey, 2003). The present of αvβ3 integrins on the endometrial surface and trophoblast facilitate embryo adhesion (Somkuti et al., 1997). Reduced expression of this molecule correlates with the infertility rate (Lessey, 1998). T also reduced the levels of expression of E-cadherin in the uterus. Down-regulation of E-cadherin has been linked to decreased number of implantation site (Liu et al., 2006). E-cadherin plays a role in mediating contact between trophectoderm and uterine epithelium (Liu et al., 2006). These findings also indicated that Muc-1 expression and distribution in the uterus were reduced following T treatment. Muc-1 is abundantly expressed at the apical surface of luminal and glandular epithelia (Brayman et al., 2004) and form a scaffold that allows the blastocyst to roll over the surface epithelium and prevents the embryo from attaching to the site other than the site destined for implantation (Carson et al., 2006). In mice, Muc-1 expression was up-regulated by E but was down-regulated during uterine receptivity period, most probably due to P. However in humans, expression of Muc-1 in the uterus was up-regulated by P (Hey et al., 1994; Aplin et al., 1998). The removal of Muc-1 would allow the embryo to adhere to the surface epithelium (Singh & Aplin, 2009).

In view, that T significantly reduces expression of αvβ3 integrin, E-cadherin and Muc-1 in the uterus, adhesion of the embryo to the endometrial surface could be impaired. Additionally, an embryo could prematurely adhere to endometrium due to reduced expression of Muc-1. These findings have also shown that levels of signalling molecules i.e. Ihh, Msx-1 and Fgf-1 in the uterus were significantly reduced following
injection of T. Ihh is expressed at high levels in uterine luminal and glandular epithelia prior to receptivity period (Paria et al., 2001) and is a major mediator for P signalling at the time of receptivity (Lee et al., 2006). Msx-1 is important in mediating embryo-endometrial contact and is crucial for embryonic development and is expressed at high levels in uterine luminal and glandular epithelia (Pavlova et al., 1994). Fgf-1 is a paracrine effector for cell proliferation and angiogenesis (Sak et al., 2013) and is involved in promoting epithelial proliferation via E signalling (Wetendorf & DeMayo, 2012). Fgf-1 has been shown to be involved in inducing activation of ERα that regulates Muc-1 expression in the endometrium (Kato et al., 1995). Conditional deletion and down-regulation of Ihh resulted in loss of receptivity that leads to implantation failure (Franco et al., 2010). Deletion of Msx-1 has also been shown to result in infertility (Nallasamy et al., 2012). In view, that expression of these signalling molecules was reduced under the influence of T, therefore, paracrine signalling in the endometrium and between endometrium and blastocyst could be impaired.

In conclusion, high dose of testosterone suppressed pinopode development, reduces complexity of tight junction and expressions of MECA-79, claudin-4, occludin, αvβ3 integrin, E-cadherin, muc-1, Msx-1, Fgf-1 and Ihh. Testosterone also reduced the number of implantation sites.
7.1 Conclusion

In this study, T was found to significantly reduce E- induced uterine fluid and Cl- secretions, cAMP levels, the size of the uterine lumen and expression and distribution of CFTR. The inhibitory effects of T might disturb the normal fluid and electrolyte composition in particular Cl- and HCO₃⁻ of the uterine fluid, which could have implications on the sperm transport, capacitation and fertilisation. Additionally, testosterone significantly induced up-regulation of Aqp-1, 5, and 7 expressions in the uterus could also disturb the normal uterine fluid volume. These findings are important since the presence of high levels of T in a condition such as polycystic ovaries (PCO) could potentially interfere with uterine fluid volume and electrolyte regulation. However, testosterone at physiological levels might be required for normal regulation of the volume of uterine fluid that is crucial for implantation.

I have shown further that high level of T could affect endometrial receptivity development in rats receiving sex-steroid replacement mimicking hormonal changes in early pregnancy. T could suppress the expression of pinopodes and L-selectin ligand (MECA-79) in the uterus, which could prevent initiation of physical contact between the blastocyst and the receptive endometrium. Additionally, T could affect the appearance of the tight junctions (TJs) where it appears less deep, less intertwining and less interconnected with decreased amount of its protein claudin-4 and occludin. Administration of T caused significantly decreased expression and distribution of αvβ3 integrin, E-cadherin and Muc-1. Collectively, these T effects could disturb development of endometrial receptivity that is crucial for blastocyst implantation.
I have shown further that effect of T could also be seen in intact rats at the early days of pregnancy. Administration of T decreased pinopodes development, altered the normal appearance of TJs and affects the expression of TJs protein i.e claudin-4 and occludin. T also reduced expression of MECA-79, αvβ3 integrin, E-cadherin and Muc-1 and endometrial signalling molecules i.e. Msx-1, Ihh and Fgf-1 in the uterus. These adverse T effects could result in reduced number of implanting embryos in the uterus of early pregnant rats.

In conclusions, this study has provided a mechanism underlying implantation failure as reported in female rats having high plasma T levels. The changes induced by T could ultimately lead to high infertility rate as seen in conditions associated with hyperandrogenemia such as polycystic ovarian disease and excessive anabolic hormone intake.

![Figure 7.1](image_url): Summary of the effects of testosterone on the uterine fluid regulation and endometrial receptivity.
7.2 Future directions

Uterine decidualisation is important in determining successful pregnancy establishment following trophoblast invasion. The implanting blastocyst will develop normally when the endometrium undergoes decidualisation process. Defective decidualisation may result in pregnancy early pregnancy loss. Decidualisation is marked by the differentiation and transformation of endometrial stroma cells to decidual cells. Multiple genes and signal molecules are involved in decidualisation process. Among these include insulin-like growth factor binding protein (IGFBP-1), bone morphogenetic protein 1 and 2 (BMP-1 and 2), Wilms tumour protein (Wt-1), cyclooxygenase-2 (COX-2), interleukin 11 (IL-11) and early growth response gene 1 (Egr1) which are the decidualization marker. Thus, future study is needed to confirm the roles of T in mediating uterine decidualisation.
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LIST OF PUBLICATIONS AND PAPERS PRESENTED


CONFERENCE PROCEEDINGS


APPENDIX A-REAGENT PREPARATION

SDS-page

1. **Acrylamide** (Acrylamide, MW: 71.08 kd and bisacrylamide, MW 154.17 kda)

   Stock concentration of acrylamide: 30%
   Stock concentration of bisacrylamide: 0.8%
   Acrylamide (30g) and bisacrylamide (0.8g) was dissolved in double distilled water. The solutions were topped up to 100ml with double distilled water and stored in dark at 4ºC.

2. **4 x resolving buffer** (ph 8.8) (Tris, MW: 121.1 kda)

   Stock solution (1.5M): 18.17g of tris was dissolved in double distilled water and volume topped up to 100ml. The solution was stored at 4ºC.

3. **4 x stacking buffer** (ph 6.8) (Tris, MW: 121.1 kdh)

   Stock solution (0.5M): 3.03g of tris was dissolved in double distilled water and volume topped up to 50ml. The solution was stored at 4ºC.

4. **Sodium dodecyl sulphate** (SDS) (MW: 288.38 kdh)

   Stock solution (10%): 5g of sodium dodecyl sulphate was dissolved in double distilled water and volume topped up to 50ml. The solution was stored at 4ºC.

5. **Ammonium persulphate** (APS) (MW : 228.2 kda)

   Stock solution (10%): 0.1g of ammonium persulphate was dissolved in double distilled water and volume topped up to 1ml. The solution was stored at 4ºC.

6. **2 x treatment buffer**

   Stock concentration of tris: 0.125M (ph 6.8)
   Stock concentration of SDS: 4%
   Stock concentration of glycerol: 20%
   Stock concentration of dithiothreitol: 0.2M
   Stock concentration of bromophenol blue: 0.02%
   Glycerol (2ml), dithiothreitol (0.31g), bromophenol blue (0.002g), SDS (4ml of 10% SDS) and tris (2.5ml of 0.5M tris) was dissolved in double distilled water. The solution were topped up to 10ml with double distilled water and stored at -20ºC.
7. **Tank buffer** (Tris, MW: 121.1 kd and glycine, MW 75.07 kDa)

- Stock concentration of tris: 0.025M (ph 8.3)
- Stock concentration of glycine: 0.192M
- Stock concentration of SDS: 0.1%

Tris (3.03g), glycine (14.41 g) and SDS (1g) was dissolved in double distilled water. The solution were topped up to 1000L with double distilled water and stored at RT.

**Western-blot**

1. **Towbin buffer**

   - Stock concentration of tris: 25mM
   - Stock concentration of glycine: 192mM
   - Stock concentration of glycine: 192mM

Tris (3.03g) and glycine (14.4g) was dissolved in double distilled water. The solution were topped up to 1L with double distilled water and stored at RT.

2. **Phosphate buffer saline**

   - Stock concentration of potassium phosphate (K$_2$PO$_4$): 
   - Stock concentration of sodium phosphate (NA$_2$HPO$_4$): 
   - Stock concentration of sodium chloride (NACl$_2$): 
   - Stock concentration of potassium chloride (KCl):

Potassium phosphate (0.12g), sodium phosphate (0.72g), sodium chloride (4.0g) and potassium chloride (0.1g) was dissolved in double distilled water. The solution were topped up to 500ml with double distilled water and stored at 4ºC.

3. **Phosphate buffer saline-tween (PBST)**

   - Stock concentration of bovine serum albumin (BSA): 1%
   - Stock concentration of tween: 0.1%

Bovine serum albumin (2.5ml) and tween (250µl) was dissolved in double distilled water. The solution were topped up to 250ml with double distilled water and stored at 4ºC.
Immunohistochemistry and Immunofluorescence

1. **4% Paraformaldehyde/PBS**
   - Phosphate Buffered Saline (PBS)---------100ml
   - Paraformaldehyde------------------------4 g

   Mix to dissolve and cover the conical flask with parafilm and transfer to fume hood. Place flask on hotplate and heat with moderate stirring. When dissolve, switch off the heat but leave to stir. When cooled transfer fixative to 4°C refrigerator.

2. **Sodium Citrate Buffer (10mM Sodium Citrate, 0.05% Tween-20, pH 6.0)**
   - Tri-sodium citrate (dihydrate) ------ 2.94 g
   - Distilled water --------------------- 1000 ml

   Mix to dissolve. Adjust pH to 6.0 with 1N HCl and then add 0.5 ml of Tween 20 and mix well. Store this solution at room temperature for 3 months or at 4°C for longer storage.

3. **Preparation Harris’ Hematoxyline**
   - Hematoxylin---------------------5gm
   - Absolute alcohol-------------50ml
   - Ammonium alum---------------100gm
   - Distilled water-----------1000ml
   - Mercuric oxide-----------2.5gm
   - Glacial acetic acid--------40ml

   Dissolve the hematoxylin in absolute alcohol and ammonium alum in hot water. Mix the two solutions and heat to boiling. Remove from flame, and add mercuric oxide and cool rapidly. Glacial acetic acid if added gives brisk nuclear staining, but life of the solution is reduced. Hence if acetic acid is to be added, it should be added in working solution.
Original Article

Testosterone decreases the expression of endometrial pinopode and L-selectin ligand (MECA-79) in adult female rats during uterine receptivity period

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Received February 28, 2014; Accepted April 3, 2014; Epub April 15, 2014; Published May 1, 2014

Abstract: Pinopode, a progesterone-dependent endometrial projection which appears during uterine receptivity period, participates in blastocyst implantation. Blastocyst loosely attaches to pinopode via L-selectin ligand (MECA-79). We hypothesized that pinopode and MECA-79 expressions were affected by testosterone. Therefore, the effect of testosterone on pinopode and MECA-79 expressions during uterine receptivity period were investigated. Methods: Ovariectomized adult female rats received 8 days sex-steroid replacement intended to mimic hormonal changes in early pregnancy with day 6 to 8 represents uterine receptivity period. Testosterone (1 mg/kg/day) was injected together with flutamide or finasteride during the period of uterine receptivity. At the end of treatment, rats were sacrificed and uteri were removed. The existence of pinopodes in the endometrium was visualized by electron microscopy and uterine expression and distribution of MECA-79 protein were examined by Western blotting and immunohistochemistry (IHC) respectively. Results: Abundant pinopodes and MECA-79 expressions were observed in rats received normal steroid replacement regime. Administration of testosterone during uterine receptivity period reduced pinopodes and MECA-79 expressions, which were antagonized by flutamide and not finasteride. Conclusions: The decrease in uterine pinopodes and MECA-79 expressions during uterine receptivity period by testosterone may cause failure of blastocyst to implant in conditions associated with high level of this hormone.

Keywords: Pinopode, testosterone, MECA-79, uterine receptivity

Introduction

Pinopodes are dome-like projections arising from the apical membrane of uterine luminal epithelia [1]. In rats, this projection appears at day 4, peaking at day 5 and rapidly decline at day 6 of early pregnancy, coincide with uterine receptivity or implantation window period [2]. Due to its existence within a limited time period, pinopode has been used as a marker of uterine receptivity [3]. The appearance of pinopodes coincides with the expression of other uterine receptivity markers such as leukemic inhibitory factor (LIF) [4], mucin, L-selectin, integrin αvβ3 and heparin-binding epidermal growth factor (HBEGF) [5, 6]. In humans however, its role as uterine receptivity marker is debatable [7].

In the uterus, pinopode displays several functions. In rodents, this projection is involved in pinocytosis which assist the removal of fluid from the uterine lumen to initiate luminal closure preceding blastocyst attachment [8]. Additionally, this bulb-like projection has been reported to participate in embryo-endometrial interaction via L-selectin ligand [9] which contains a carbohydrate epitope, MECA-79 [10]. In humans, pinopodes were found to release secretory vesicles containing LIF into the uterine lumen [11]. Pinopode expression is progesterone dependent [12]. A strong correlation between pinopode with plasma P2 level and P4 receptor expression has been reported in the uterus of humans [12], rats [1] and mice [13]. Estrogen however causes loss of pinopode expression [1]. Ovariectomized mice were found...